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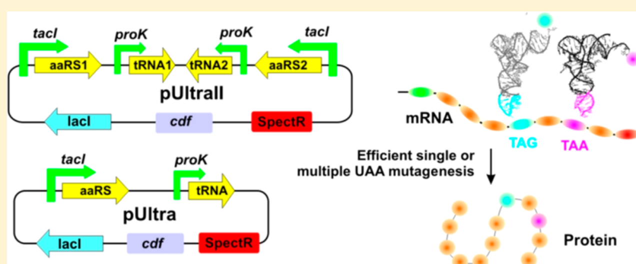
A Versatile Platform for Single- and Multiple-Unnatural Amino Acid Mutagenesis in *Escherichia coli*

Abhishek Chatterjee, Sophie B. Sun, Jennifer L. Furman, Han Xiao, and Peter G. Schultz*

Department of Chemistry and Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037, United States

Supporting Information

ABSTRACT: To site-specifically incorporate an unnatural amino acid (UAA) into target proteins in *Escherichia coli*, we use a suppressor plasmid that provides an engineered suppressor tRNA and an aminoacyl-tRNA synthetase (aaRS) specific for the UAA of interest. The continuous drive to further improve UAA incorporation efficiency in *E. coli* has resulted in several generations of suppressor plasmids. Here we describe a new, highly efficient suppressor plasmid, pUltra, harboring a single copy each of the tRNA and aaRS expression cassettes that exhibits higher suppression activity than its predecessors. This system is able to efficiently incorporate up to three UAAs within the same protein at levels up to 30% of the level of wild-type protein expression. Its unique origin of replication (*CloDF13*) and antibiotic resistance marker (spectinomycin) allow pUltra to be used in conjunction with the previously reported pEVOL suppressor plasmid, each encoding a distinct tRNA/aaRS pair, to simultaneously insert two different UAAs into the same protein. We demonstrate the utility of this system by efficiently incorporating two bio-orthogonal UAAs containing keto and azido side chains into ketosteroid isomerase and subsequently derivatizing these amino acid residues with two distinct fluorophores, capable of Förster resonance energy transfer interaction. Finally, because of its minimal composition, two different tRNA/aaRS pairs were encoded in pUltra, allowing the generation of a single plasmid capable of dual suppression. The high suppression efficiency and the ability to harbor multiple tRNA/aaRS pairs make pUltra a useful system for conducting single- and multiple-UAA mutagenesis in *E. coli*.



The ability to site-specifically incorporate unnatural amino acids (UAAs) into proteins in living cells significantly improves our ability to manipulate protein structure and function.[1] An engineered tRNA/aminoacyl-tRNA synthetase (aaRS) pair is used to insert the UAA of interest in response to a nonsense or frameshift codon. In *Escherichia coli*, two different tRNA/aaRS pairs (*Methanocaldococcus jannaschii* tyrosyl and *Methanosarcina barkeri* and *Methanosarcina mazei*-derived pyrrolysyl) have been engineered to genetically encode a large number of UAAs with a variety of novel functions, including amino acids with unique chemical reactivities, as well as metal binding, photo-cross-linking, photocaged, and fluorescent amino acids.[1] While this technique has been expanded to *Saccharmoyces cerevisiae*,[2] *Pichia pastoris*,[3] and mammalian cells,[4−7] *E. coli* remains an attractive expression host because of its robustness, facile manipulation, high levels of protein expression, and the large set of available genetically encoded UAAs.[1] To express a protein of interest containing a desired UAA at a specific site in *E. coli*, an expression plasmid encoding its corresponding nonsense or frameshift mutant is cotransformed with a suppressor plasmid that harbors the tRNA/aaRS pair, specific for the desired UAA. An early version of this suppressor plasmid employed an *lpp* promoter and a *glnS* promoter to express the tRNA and aaRS, respectively.[8] To improve the suppression efficiency of this system, a second-generation suppressor plasmid pSup[9] was developed with an enhanced *glnS′* promoter to increase the level of synthetase expression and six copies of the tRNA expression cassette under the *proK* promoter. A variation of this plasmid, pSUPAR,[10] that harbored an additional *araBAD*-driven aaRS expression cassette and three copies of the tRNA driven by *proK* was also generated. Further optimization of this system yielded pEVOL,[11] which harbored one copy of the tRNA expression cassette under the efficient *proK* promoter and two copies of the aaRS under *araBAD* and *glnS* promoters (Figure 2a). This latter vector resulted in a substantial improvement in suppression efficiency, approaching expression levels of the wild-type protein (without nonsense suppression), when used in conjunction with the efficient tRNA/aaRS pairs derived from the *M. jannaschii* tyrosyl (MjTyr) system.[11] However, when less efficient tRNA/aaRS pairs are used, the suppression efficiency of pEVOL decreases, indicating possible room for improvement.

In addition to the incorporation of a single UAA, the availability of mutually orthogonal tRNA/aaRS pairs, suppressing distinct codons in *E. coli*, has made possible the site-specific incorporation of multiple, different UAAs into the same

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protein.[12−16] To express proteins containing multiple, distinct UAAs, the relevant suppressor tRNA/aaRS pairs must be co-expressed with the target gene containing appropriately positioned nonsense and/or frameshift codons specifying the sites for UAA insertion. An efficient suppressor plasmid that allows the expression of multiple tRNA/aaRS pairs would facilitate the incorporation of multiple UAAs; however, the requirement for multiple copies of the tRNA or aaRS expression cassettes in the existing suppressor plasmids significantly complicates the integration of multiple tRNA/aaRS pairs within the same vector.

Here we report the construction of a new suppressor plasmid, pUltra, which uses a single copy each of the tRNA and aaRS expression cassettes yet exhibits enhanced suppression efficiency relative to that of its most efficient counterpart, pEVOL. Using a pUltra plasmid expressing a tRNA/aaRS pair derived from the MjTyr system, suppression of three different amber stop codons within GFP was achieved with good efficiency. The pyrrolysyl-tRNA/aaRS pair was optimized for enhanced ochre (TAA) suppression and was also encoded in the pUltra plasmid. Together with the amber (TAG) suppressing MjTyr-tRNA/aaRS pair encoded in the pEVOL plasmid, this system was used to incorporate two different UAAs into the same protein in good yields. Furthermore, the simple composition of pUltra facilitated the generation of a dual suppressor plasmid that encodes both the ochre suppressing *M. barkeri* pyrrolysyl and the amber suppressing *M. jannaschii* tyrosyl tRNA/aaRS pairs.

■ MATERIALS AND METHODS

The DH10B (Life Technologies, Carlsbad, CA) strain of *E. coli* was used to clone or propagate plasmid DNA. The Qiagen (Valencia, CA) spin miniprep kit was used to purify plasmid DNA, while Macherey-Nagel (Bethlehem, PA) Nucleospin columns were used to purify DNA following digestion or gel electrophoresis. Platinum *pfx* DNA polymerase (Life Technologies) was used to perform polymerase chain reaction (PCR). Agilent QuikChange Lightning Site-Directed or Multi Site-Directed mutagenesis kits were used to generate single or multiple site-directed mutations, respectively. DNA oligomers for PCRs were purchased from IDT (Coralville, IA). The list of oligomers used in this report can be found in the Supporting Information. Restriction enzymes and T4 DNA ligase from NEB (Ipswich, MA) were used. Antibiotics, isopropyl β-D-1-thiogalactopyranoside (IPTG), and L-arabinose were purchased from Sigma-Aldrich (St. Louis, MO) and Fisher Scientific (Waltham, MA). Alexa Fluor 488 C_5-aminooxyacetamide, Click-IT Alexa Fluor 488 DIBO Alkyne, and Alexa Fluor 594 DIBO Alkyne were obtained from Life Technologies. Bacteria were grown on LB/agar plates and 2×YT or TB liquid medium with the following antibiotic concentrations unless otherwise mentioned: 50 μg/mL kanamycin, 50 μg/mL spectinomycin, 50 μg/mL chloramphenicol, and 100 μg/mL ampicillin. Components of media were obtained from BD-Difco (Franklin Lakes, NJ) and Corning-Mediatech, Inc. (Manassas, VA). Synthesis of the MbPylRS gene with codon optimization for *E. coli* was conducted by GenScript (Piscataway, NJ).

Construction of pUltra, pEVOL*tac*, and pUltraII Vectors. The *Clodf13* replicon, spectinomycin resistance cassette and the *lacI* gene of pUltra were derived from pCDF-1b. To construct pUltra, the T7 promoter/terminator elements in pCDF-1b (Novagen) were first replaced with a *tacI* promoter sequence followed by a multiple-cloning site. This was achieved by incorporating the corresponding insert DNA between the MluI and BsaAI restriction sites of pCDF-1b. The insert DNA was generated by overlap-extension PCR of two fragments, obtained using primers CDF-Mlu-F/CDFi-R and CDF-Bsa-R/CDFi-F and pCDF-1b as the template. Next, the rrnB terminator element was amplified via PCR from pEVOL using primers rrnB-Not-F and rrnB-Sbf-R, and inserted between the NotI and SbfI restriction sites downstream from the *tacI* promoter, to generate pUltra-basic. The proK-$tRNA_{CUA}^{MjTyr}$ cassette was amplified from pEVOL using primers proK-F and proK-R and inserted between the SbfI and NcoI restriction sites of pUltra-basic. Inserts for other tRNAs were generated by overlap-extension PCR of two fragments, obtained using this plasmid as the template and a combination of primers proK-F/tRNA-R and proK-R/tRNA-F. These fragments were then inserted into pUltra-basic using the same strategy. Following the introduction of the tRNA into pUltra-basic, the corresponding aaRS was amplified using primers RS-F and RS-R and inserted using the NotI restriction site downstream of the *tacI* promoter to generate the corresponding pUltra. To generate pUltra-Pro, *Pyrococcus horikoshii* ProRS variant $PhPRS_{CUA}$-h1 was used.[23] For pUltra-Trp, *S. cerevisiae* $Trp\text{-}tRNA_{AS3.5}$ was used along with wild-type *Sc*TrpRS. The ochre suppressor pUltra-pyrrolysine was generated by site-directed mutagenesis of the corresponding amber suppressor vector using primers MmPylT-TAA-F and MmPylT-TAA-R. The U25C mutant of MmPylT was generated by site-directed mutagenesis using primers MmPylT-U25C-F and MmPylT-U25C-R.

To construct pEVOL*tac*, a DNA insert encoding the *lacI* gene, the *tacI* promoter, and *rrnB* terminator elements was amplified from pUltra-basic (using primers U2Ev-F and -R) and was inserted into the pEVOL plasmid using restriction sites HindIII and XhoI (removing the endogenous synthetase expression cassettes and the *araC* gene encoded in pEVOL).

To construct pUltraII, the aaRS and the tRNA expression cassettes were amplified from pUltra-MjTyr (pAcF-specific) using primers *tacI*-SalI-F and tRNA-XhoI-R. This DNA fragment was digested with SalI and XhoI and inserted into the XhoI restriction site of pUltra-Pyl^{TAA} (encoding MbPylRS^{opt} and $tRNA_{UUA}^{PylU25C}$).

Construction of Expression Plasmids. pET101-GFP (wild type and Tyr151TAG) was constructed as described previously.[11] The triple amber mutant of GFP was constructed by site-directed mutagenesis of pET101-GFP using gfpTAGm-3 and gfpTAGm-151.153. GFP-3TAG-151TAA was generated by site-directed mutagenesis of pET101-GFPwt using gfpTAGm-3 and gfpTAAm-151.

Analysis of Aminoacyl-tRNA Synthetase Expression. pBK-*tacI*-MjTyrRS-$(His)_6$, pBK-*tacI*-MbPylRS-$(His)_6$, and pBK-*tacI*-MbPylRS(opt)-$(His)_6$ were transformed into DH10B *E. coli* by electroporation. An overnight culture of these cells was used to inoculate 25 mL of 2×YT medium supplemented with 50 μg/mL kanamycin at an OD_{600} of 0.05. At an OD_{600} of 0.7, the cultures were cooled from 37 to 30 °C for 16−24 h. Cells were harvested by centrifugation and lysed using BugBuster Protein Extraction Reagent (EMD-Millipore, Billerica, MA) supplemented with Complete Mini EDTA-free protease inhibitor cocktail (Roche, Indianapolis, IN), 0.5 mg/mL lysozyme (MP Biomedicals, Solon, OH), and 5 units/mL Benzonase Nuclease (Novagen) according to the manufacturer's instructions. Following lysis, cell debris was removed by centrifugation at 18000*g*. Cell free extract, the insoluble

fraction, and the soluble fraction were separated via reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Novex 4 to 20% Tris-glycine, Invitrogen), transferred to nitrocellulose, and probed with a 1:1000 dilution of anti-His_6-HRP (Clontech, Mountain View, CA). Blots were developed using SuperSignal West Dura Chemiluminescent substrate (Pierce) and exposed to film.

Expression, Purification, and Mass Spectrometry (MS) Analyses of Reporter Proteins. GFP expression was performed as described previously.[11] In summary, *E. coli* BL21(DE3) carrying the expression plasmid and the suppressor plasmid (pEVOL, pEVOL*tac*, pUltra, or pUltraII) was grown in rich medium (2×YT or TB) until the OD_{600} reached 0.6–0.8, at which point the expression of the target gene and the synthetase were co-induced with 1 mM IPTG (for pEVOL*tac*, pUltra, and pUltraII) or 1 mM IPTG and 0.02% L-arabinose (for pEVOL). The relevant UAAs were also added at this point to a final concentration of 1 mM. Protein expression was continued for 10–14 h at 37 °C. For rapid analysis of GFP expression, cells from a 100 μL culture were resuspended in 200 μL of PBS (Mediatech) and transferred to 96-well black clear bottom assay plates, and GFP fluorescence was measured using a SpectraMax GeminiEM plate reader (Molecular Devices). Fluorescence readings were normalized by dividing by OD_{600}.

To isolate overexpressed proteins, cells from a 10–40 mL culture were harvested by centrifugation, resuspended in 0.5–2 mL of BugBuster Protein Extraction Reagent supplemented with Complete Mini EDTA-free protease inhibitor cocktail, 0.5 mg/mL lysozyme, and 5 units/mL Benzonase Nuclease, and incubated on ice for 15 min. The lysate was clarified by centrifugation at 18000*g* for 15 min, and the His_6-tagged protein (C-terminal) was isolated using Qiagen Ni-NTA Agarose Superflow resin according to the manufacturer's instructions. The molecular weights of purified proteins were verified using electrospray ionization mass spectrometry (ESI-MS) analysis.

Site-Specific Conjugation of Alexa Fluor 488 to GFP-pAcF3-AzK151. GFP-3TAG-151TAA was expressed from the pET101 plasmid, and pEVOL-pAcF and pUltra-Pyl^{TAA} were used to suppress the amber and ochre codons with pAcF and AzK, respectively. Protein expression and purification were performed as described above. For the ketone–hydroxylamine conjugation reaction, the purified protein was concentrated to 60 μM and buffer-exchanged into 100 mM sodium acetate buffer (pH 4.5) using Micro Bio-Spin 6 desalting columns (Bio-Rad, Hercules, CA), following the manufacturer's instructions. To 25 μL of protein were added Alexa Fluor 488 C_5-aminooxyacetamide and *p*-methoxyaniline at final concentrations of 300 μM and 100 mM, respectively. For the azido–cyclooctyne conjugation reaction, the purified protein was concentrated to 60 μM and buffer-exchanged into DPBS (Gibco) using Micro Bio-Spin 6 desalting columns (Bio-Rad), following the manufacturers' instructions. To 25 μL of protein was added Click-IT Alexa Fluor 488 DIBO Alkyne at a final concentration of 300 μM. Both reactions were allowed to proceed overnight (14 h) at 37 °C in the dark, and the mixtures were subsequently resolved by SDS–PAGE. Protein bands were detected by Coomassie staining, while fluorescence was detected using UV transillumination using a Bio-Rad ChemiDoc XRS imaging system.

Site-Specific Double Labeling of KSI-AzK7-pAcF78 with Alexa Fluor 488 and 594. KSI-7TAA-78TAG was expressed from the pET28 plasmid using pUltra-Pyl^{TAA} and pEVOL-pAcF to suppress the ochre and the amber codons with AzK and pAcF, respectively. Protein expression and purification were performed as described above. The purified protein was buffer-exchanged into 100 mM sodium acetate buffer (pH 4.5) using an Amicon Ultra-15 3 kDa molecular weight cutoff (MWCO) membrane filter and concentrated to 50 μM. To 100 μL of this protein was added Alexa Fluor 488 C_5-aminooxyacetamide at a final concentrations of 500 μM, and the conjugation reaction was allowed to proceed overnight at 37 °C in the dark. The protein–dye conjugate precipitated under the reaction conditions, was washed with PBS, and was dissolved in 100 μL of 8 M urea. At this point, Alexa Fluor 594-DIBO was added at a final concentration of 300 μM to the protein, and the reaction mixture was incubated overnight at 37 °C in the dark. The doubly labeled protein was refolded via rapid dilution into 15 mL of ice-cold DPBS containing 5 mM β-mercaptoethanol and overnight incubation in the dark at 4 °C. The protein was concentrated, and the excess fluorophore was removed using an Amicon Ultra-15 3 kDa MWCO membrane filter. Doubly labeled protein was analyzed by MS, absorbance, and fluorescence spectroscopy.

■ RESULTS AND DISCUSSION

Construction of pEVOL*tac* and pUltra. The expression levels of the suppressor tRNA and the corresponding aaRS can be modulated by the choice of the promoters used to drive their transcription and the number of copies of each expression cassette encoded in the suppression plasmid. During the construction of pEVOL, the most efficient suppressor plasmid available to date,[11] it was found that a single expression cassette of the tRNA driven by the efficient *proK* promoter provides robust suppression efficiency, which did not significantly improve upon the inclusion of additional copies of the tRNA gene. However, the presence of two expression cassettes of the aaRS, expressed from a constitutive weak promoter (*glnS*) and an inducible strong promoter (*araBAD*), was crucial for achieving high suppression levels.[11] Some inducible promoters allow low levels of leaky expression in the absence of the inducer,[17,18] which may mimic the expression pattern of the aaRS from pEVOL. One promising candidate is the *tacI* promoter,[19] a hybrid of *lac*UV and *trp* promoters, which can be repressed by LacI and can be derepressed with IPTG. Like *lac*UV, this promoter shows residual leaky activity in the absence of the inducer (IPTG), especially in a rich culture medium (e.g., LB). We replaced the two aaRS expression cassettes of pEVOL with a single copy of the *tacI*-driven expression cassette of an MjTyrRS variant, specific for *p*-acetylphenylalanine [pAcF (Figure 1)], to generate suppressor plasmid pEVOL*tac* (Figure 2a). A strong ribosome binding site sequence (AAGGAG) was inserted preceding the start codon, and the *rrnB* transcription–termination sequence from pEVOL was introduced following the aaRS. The original *proK*-driven Mj-$tRNA_{CUA}^{Tyr}$ expression cassette in pEVOL was retained in the new plasmid. Also, repressor element *araC* in pEVOL was replaced with *lacI* to allow IPTG inducible expression of the aaRS from the *tacI* promoter.

Traditionally, the suppressor plasmid is constructed using a low-copy number *p15a* (10–12 copies per cell)-derived origin of replication (ori) to allow compatibility with *colE1*- or *pBR322*-derived origins, which is used in the vast majority of the common *E. coli* protein expression vectors (e.g., pET, pBAD, pQE, etc.). Other ori's that are mutually compatible

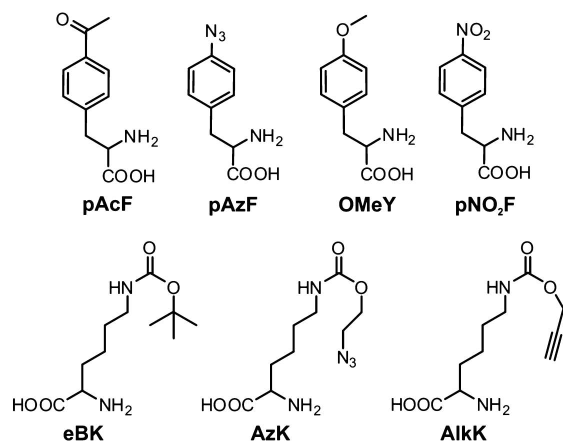

Figure 1. UAAs used in this study.

with both *p15a* and *colE1* are now available.[20,21] A suppressor plasmid that uses such a rare ori would further expand its compatibility with commonly used expression vectors and facilitate its comaintenance with two additional, distinct plasmids, required for specific applications. Furthermore, *CloDF13* (*cdf*),[20] one such available ori, maintains a higher copy number (20−40 copies per cell) in *E. coli*, which may enhance the expression level of the tRNA/aaRS pair and, consequently, the suppression efficiency. Therefore, a second suppressor plasmid, pUltra, was generated by inserting expression cassettes *tacI*-MjYRS (pAcF) and *proK*-$tRNA_{CUA}^{Tyr}$ into the pCDF vector, which encodes the *cdf* ori, a spectinomycin antibiotic resistance gene and a LacI expression cassette for inducible expression of the aaRS from the *tacI* promoter (Figure 2a).

Evaluation of the Suppression Efficiencies of pEVOL-*tac* and pUltra. The previously reported GFP (Tyr151-TAG)[11] mutant was used to evaluate the suppression efficiencies of the new suppressor plasmids. Upon suppression of the amber stop codon, full-length GFP is produced, allowing rapid assessment of suppression levels based on GFP fluorescence. Also, the inherently low suppression efficiency of this mutant provides a broader dynamic range for the optimization of the suppression machinery. The new suppressor plasmids, pEVOL*tac* and pUltra, harboring MjTyrRS (pAcF) and $tRNA_{CUA}^{Tyr}$, were cotransformed with pET101-GFP (Tyr151TAG) into the BL21(DE3) strain of *E. coli*, and the expression level of GFP was analyzed in the presence and absence of pAcF in terrific broth (TB). For comparison, the corresponding pEVOL vector was also used to express the same GFP mutant, using optimal expression conditions reported above.

The suppression efficiencies of pEVOL and pEVOL*tac* were comparable, suggesting that the single *tacI*-driven aaRS expression cassette can efficiently replace the two expression cassettes in pEVOL (Figure 2b). The expression level afforded by pUltra was approximately 30% higher than both, possibly because of the higher copy number sustained by its ori (Figure 2b). Recently, a polyspecific MjTyrRS has been described,[22] originally selected to charge *p*-cyanophenylalanine (pCNF), which can be used to incorporate a large number of UAAs into target proteins. This synthetase was inserted into pUltra, and the ability of the corresponding suppressor plasmid to suppress the GFP-Tyr151TAG mutant was verified using pAcF, *O*-methyltyrosine [OMeY (Figure 1)], *p*-azidophenylalanine [pAzF (Figure 1)], and *p*-nitrophenylalanine [pNO$_2$-F (Figure 1)]. In each case, the GFP expression level was compared to that obtained with the corresponding pEVOL plasmid (Figure 2c). As observed with the pAcF-specific suppressor systems, pUltra consistently provided higher suppression levels (between 20 and 50%). We also monitored the postinduction

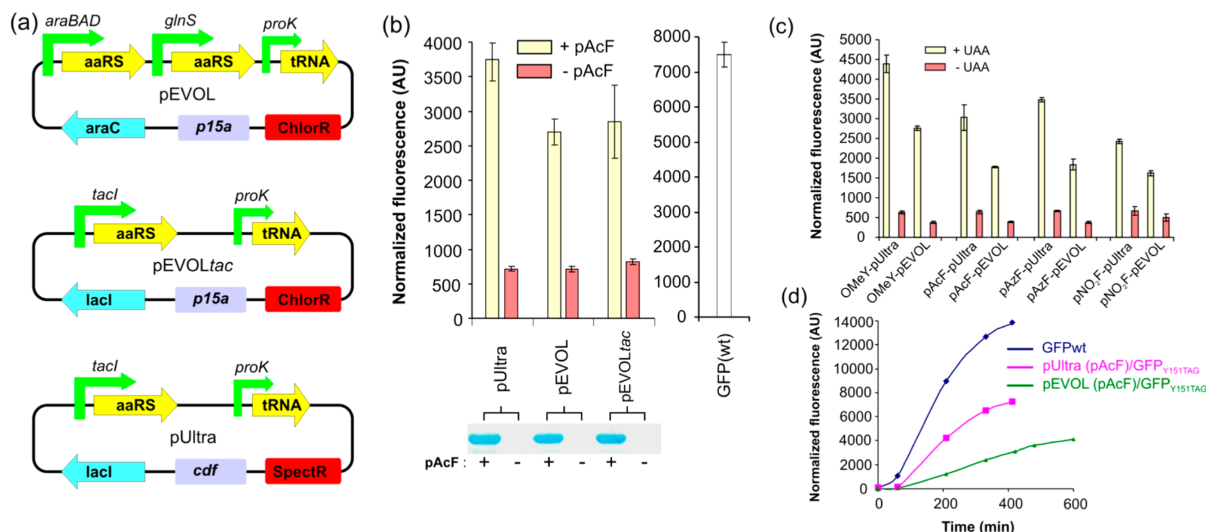

Figure 2. Vector construction and evaluation of suppression efficiency. (a) Vector maps for pEVOL, pEVOL*tac*, and pUltra. (b) Comparison of suppression efficiencies of pUltra, pEVOL, and pEVOL*tac* (all harboring the pAcF-specific MjTyr-tRNA/aaRS pair) as measured by their ability to support expression of full-length GFP from expression vector pET101-GFP(Tyr151TAG). The top panel shows normalized fluorescence in the presence (yellow) or absence (red) of pAcF; the bottom panel shows the SDS−PAGE analysis of the isolated protein from each expression experiment. wtGFP expression (without amber suppression) is shown for reference. (c) Comparison of suppression efficiencies of pUltra and pEVOL, both harboring the polyspecific MjTyr-tRNA/aaRS pair, as measured by the normalized fluorescence of full-length GFP expressed from GFP(Tyr151TAG), in the presence or absence of four different UAAs. (d) Time-dependent, postinduction expression of full-length GFP, measured as normalized fluorescence, from pET-GFP [wild type, without amber suppression (blue)] or from GFP(Y151TAG) using pUltra-pAcF (magenta) or pEVOL-pAcF (green) to suppress the amber stop codon.

expression levels of GFP (Tyr151TAG) in a time-dependent manner, using pEVOL and pUltra encoding the pAcF-specific MjTyrRS. Significantly faster expression kinetics was observed with pUltra (Figure 2d). The expression level of GFP reached saturation within 5−7 h of induction using pUltra for amber suppression, compared to 10−14 h for pEVOL.

The amber suppression efficiencies exhibited by pEVOL vectors encoding MjTyr-derived tRNA/aaRS pairs are high, approaching the wild-type protein expression levels (without amber suppression) in some instances.[11] Consequently, the dynamic range for the evaluation of the efficiency of new suppressor vectors is limited. Therefore, a new GFP expression cassette was generated carrying three amber mutations at permissive sites (GFP-3*). Only when all three amber codons are suppressed will full-length GFP be produced. When we expressed this construct using pEVOL and pUltra plasmids, both encoding the aforementioned pAcF-specific or poly-specific MjTyrRS (tested with OMeY), 200−300% improvements in expression levels were observed for pUltra (Figure 3a). This difference in suppression efficiency between the two suppressor plasmids is significantly greater than that observed using the GFP construct with a single amber codon (Tyr151TAG) and demonstrates the higher efficiency of pUltra relative to that of pEVOL. The protein was purified, and incorporation of three UAAs was confirmed by MS analysis.

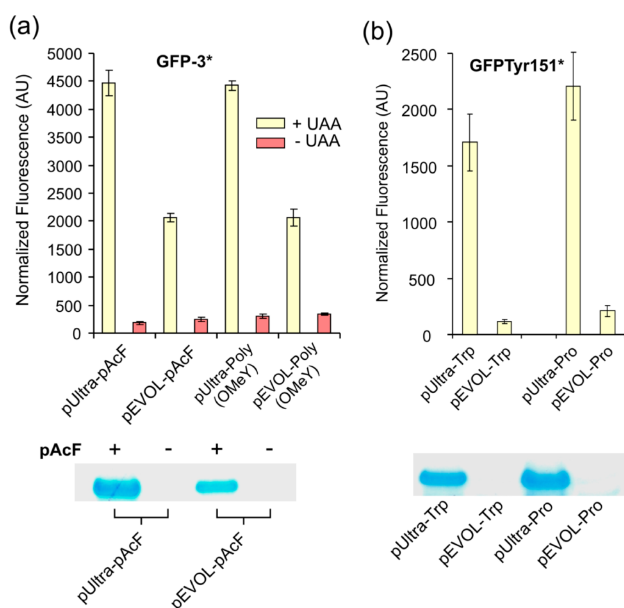

Figure 3. Comparison of suppression efficiencies of pUltra and pEVOL for GFP-3* using the MjTyr pair or for GFP-151TAG using the less efficient tRNA/aaRS pairs. (a) Expression of full-length GFP from pET-GFP-3* with three in-frame amber stop codons, using pUltra or pEVOL (encoding either a pAcF-specific or a polyspecific MjTyr tRNA/aaRS pair). The top panel shows GFP expression as normalized fluorescence, in the presence or absence of the corresponding UAA (OMeY was used for the polyspecific synthetase). The bottom panel shows the SDS−PAGE analysis of isolated protein expressed using the pAcF-specific pEVOL or pUltra in the presence (+) or absence (−) of pAcF. (b) Expression levels of GFP from pET-GFP(Y151TAG) using the pUltra or pEVOL vector encoding the engineered tryptophanyl or prolyl suppressor tRNA/aaRS pairs (charging tryptophan or proline, respectively). The top panel shows normalized fluorescence and the bottom panel SDS−PAGE analysis of the isolated protein from each expression experiment.

Yields for GFP-3* with pUltra-pAcF (∼5 mg/L) were approximately 30% of the same for wtGFP (without amber suppression).

The suppression efficiencies of pEVOL and pUltra were further evaluated with two other tRNA/aaRS pairs recently adapted in *E. coli* for amber suppression: an archaebacterium-derived prolyl-tRNA (*Archaeoglobus fulgidus*)/ProRS (*P. horikoshii*) pair[23] and a *S. cerevisiae*-derived tryptophanyl-tRNA/TrpRS pair.[24] These systems are significantly less efficient than the optimized MjTyr-derived pair, and when expressed from pEVOL, they afford low expression levels of GFP (Tyr151TAG) upon amber suppression (<5% of wtGFP). A 10−15-fold improvement in suppression efficiency was observed (10−15-fold) when the corresponding pUltra plasmids encoding the prolyl or tryptophanyl pair were used to express the same GFP construct in either case (Figure 3b). These results suggest that pUltra is a more efficient suppressor system than pEVOL and is particularly useful for UAA mutagenesis of weakly suppressing target proteins or when a weak suppressor tRNA/aaRS pair is used.

Finally, the suppression efficiencies of the pUltra plasmids encoding the pAcF-specific and polyspecific MjYRS were further verified with T4 lysozyme (Asn68TAG) and keto-steroid isomerase (His78TAG) expression plasmids. Efficient protein expression was observed in both cases, and the yields were approximately 50% relative to the corresponding wild-type proteins [without amber suppression (Figure S1 of the Supporting Information)]. All protein expressions were performed in shake flasks in 2×YT medium.

Optimization of an Ochre Suppressor Pyrrolysyl tRNA/aaRS Pair. The archaebacterium-derived pyrrolysine tRNA/aaRS pair has been engineered to genetically encode a large number of UAAs.[1] It was shown that this pair is anticodon permissive[25] and efficiently suppresses ochre (TAA) codons.[14,15] It is also orthogonal to the amber-suppressing MjTyr-derived tRNA/aaRS pairs and can be used together to incorporate two different UAAs into the same protein.[14,15] The use of this dual-suppression system should be facilitated by pUltra, which encodes a unique ori and antibiotic resistance marker and should allow the simultaneous use of two different suppression vectors for the introduction of two distinct UAAs into a target protein. The pyrrolysyl (ochre) and MjTyr (amber) suppression systems can be encoded by pUltra (*cdf* ori, spectinomycin marker) and pEVOL/pEVOl*tac* (*p15a* ori, chloramphenicol marker), respectively, and the target gene can be encoded in a common expression vector (*colE1* ori, antibiotic selection marker other than spectinomycin and chloramphenicol); all three plasmids can be comaintained in *E. coli*. The modular nature of this dual-suppression system eliminates the need to generate complicated expression plasmids.

To evaluate the suppression efficiency of the pyrrolysyl system, we constructed pUltra plasmids harboring amber suppressor $tRNA_{CUA}^{Pyl}$ (*M. mazei*)/PylRS (*M. barkeri*) pair or its ochre suppressor counterpart, Mm-$tRNA_{UUA}^{Pyl}$/MbPylRS. The suppression efficiencies of these plasmids were tested with the GFP (Tyr151TAG or TAA) expression vectors using ε-tBoc-lysine [eBK (Figure 1)], a UAA that is efficiently charged by MbPylRS.[1] While the GFP expression levels in both cases were similar, the suppression efficiencies were found to be nearly 10-fold lower than the same observed for the MjTyr-derived suppressor pairs (Figure 4c). One of the factors contributing to this lower activity was found to be the lower

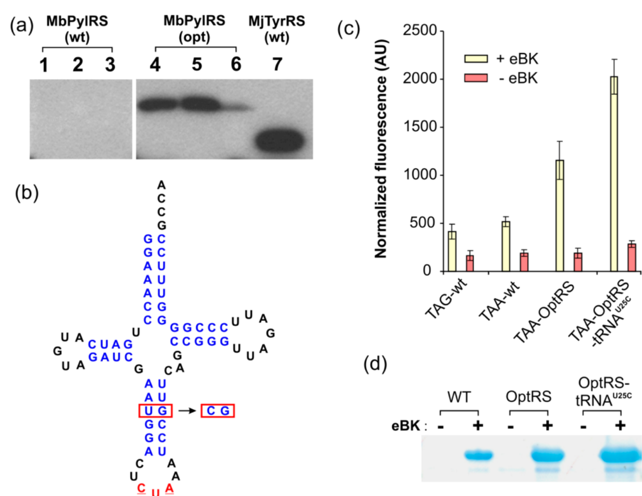

Figure 4. Optimization of the pyrrolysyl pair. (a) Codon optimization improves the expression level of MbPylRS. The His_6-tagged aaRS gene under the *tacI* promoter is expressed in *E. coli*, and protein in the lysate is detected by Western blotting. In lanes 1−3, for the wild-type MbPylRS gene, the abundance of synthetase in the crude lysate, the insoluble fraction, and the soluble fraction, respectively, is shown. In lanes 4−6, for the codon-optimized MbPylRS gene, the abundance of synthetase in the crude lysate, the insoluble fraction, and the soluble fraction, respectively, is shown. Lane 7 shows the expression level of the wild-type His_6-tagged MjTyrRS in a similar experiment for reference. (b) Pyrrolysyl-tRNA contains an unfavorable U:G wobble pair in the anticodon stem, which was mutated to a C:G pair to improve suppression efficiency. (c) Suppression efficiencies of different pyrrolysyl-tRNA/aaRS pairs (in pUltra), measured as the expression level of GFP (normalized fluorescence) from pET-GFP(Y151TAG) or pET-GFP(Y151TAA) in the presence (yellow) or absence (red) of eBK. TAG-wt and TAA-wt represent pUltra vectors harboring amber- and ochre-suppressing wild-type pyrrolysine pairs, respectively. TAA-OptRS represents the combination of wild-type ochre suppressor tRNA and codon-optimized MbPylRS, while TAA-OptRS-tRNAU25C represents the combination of the U25C mutant of the ochre suppressor tRNA and codon-optimized MbPylRS. (d) Isolated protein samples expressed from pET-GFP(Y151TAA) using pUltra vectors, encoding the three different combinations of ochre-suppressing pyrrolysyl pairs described above, in the presence (+) or absence (−) of eBK.

expression levels of MbPylRS compared to those of MjTyrRS. When MjTyrRS was expressed from the *tacI* promoter, strong expression could be observed using a Western blot (Figure 4a, lane 7), while the same experiment with MbPylRS failed to detect any protein (Figure 4a, lanes 1−3). Replacing the original copy of the MbPylRS gene with a variant codon-optimized for expression in *E. coli* resulted in improved expression levels (Figure 4a, lanes 4−6). When introduced into pUltra in conjunction with the ochre-suppressing Mm-$tRNA_{UUA}^{Pyl}$, MbPylRS(opt) exhibited a ∼2-fold increase in its suppression efficiency relative to that of pUltra harboring the original MbPylRS gene (Figure 4). It is worth noting that the increase in synthetase expression levels did not correlate with the increase in suppression efficiency. This may be due to improper folding of MbPylRS in *E. coli*, leading to an inactive protein. The majority of the expressed MbPylRS was found in the insoluble fraction (Figure 4a, lane 5), consistent with this hypothesis.

To further enhance the suppression efficiency of this pair, we attempted to improve the activity of $tRNA^{Pyl}$. It has previously been shown that the presence of the G:U wobble pair in the stem regions of a tRNA leads to suboptimal activity, which is often improved by a G:C substitution.[26] For the Mm-$tRNA^{Pyl}$, we identified such a G:U wobble pair in the anticodon stem (Figure 4b). A U25C mutant of $tRNA_{UUA}^{Pyl}$ was generated, substituting this wobble pair with a G:C pair, and incorporated into pUltra along with the MbPylRS(opt). When the suppression efficiency of this vector was tested with the aforementioned GFP (Tyr151TAA) expression construct, a further 2-fold increase in suppression efficiency was observed relative to that of the corresponding pUltra plasmid with wild-type $tRNA_{UUA}^{Pyl}$ (Figure 4c,d). The suppression efficiency of this optimized pyrrolysyl pair, comprising MbPylRS(opt) and $tRNA_{UUA}^{Pyl}$(U25C), is significantly improved relative to that of its wild-type counterparts and should be useful for enhanced expression of mutant proteins.

A Facile System for Suppression with Two Distinct UAAs. The development of a pUltra suppressor plasmid encoding an improved ochre-suppressing pyrrolysyl pair should facilitate the incorporation of two distinct UAAs into the same protein. To investigate the potential of this system, we generated a GFP expression plasmid that encodes a GFP-3TAG-151TAA double mutant. This plasmid was cotransformed with pUltra (encoding $MbPylRS^{opt}$ and $tRNA_{UUA}^{PylU25C}$; pUltra-Pyl^{TAA}) and pEVOL (encoding a pAzF-specific MjTyrRS/$tRNA_{CUA}^{Tyr}$) suppressor plasmids into strain BL21(DE3). The ability of the resulting bacteria to express full-length GFP by dual suppression was investigated in rich medium. No detectable GFP was produced when neither of the UAAs or only pAzF was present. Low levels of expression could be observed in the presence of eBK alone (Figure 5a). This is consistent with the observation that in the absence of its cognate UAA, MjTyr-derived tRNA/aaRS pairs exhibit higher background suppression levels than their pyrrolysine counterparts. However, when the cognate UAA is present, the fidelity of incorporation of the UAA into the target protein by the MjTyr pair is high. In the absence of eBK, little background suppression is observed with the highly orthogonal

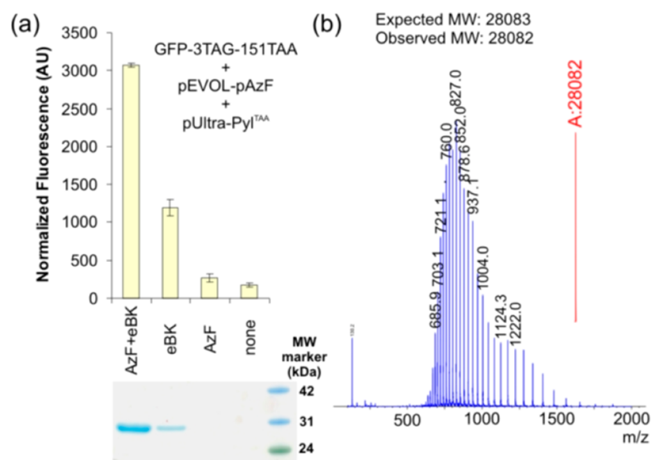

Figure 5. Simultaneous incorporation of two different UAAs into GFP. Full-length GFP was produced from pET-GFP-3TAG-151TAA using pEVOL-pAzF to suppress TAG with pAzF and pUltra-Pyl^{TAA} to suppress TAA with eBK. (a) Normalized fluorescence (top) and isolated protein by SDS−PAGE analysis (bottom) in the presence of both UAAs (AzF and eBK), eBK alone, or pAzF alone or in the absence of both UAAs (none). (b) ESI-MS analysis of the purified protein reveals a homogeneous species with the correct molecular weight.

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pyrrolysyl pair. Robust production of GFP (6 mg/L; ~33% of that of wtGFP) was observed only in the presence of both pAzF and eBK (Figure 5a). The protein was isolated using a C-terminal hexahistidine tag and characterized by ESI-MS analysis, which revealed a homogeneous species, incorporating both pAzF and eBK (Figure 5a,b). Wild-type MbPylRS is also able to accept several other UAAs, including AzK and AlkK (Figure 1), which contain functional groups allowing bio-orthogonal conjugation reactions. Using the same conditions described above, pAzF and AlkK were also incorporated into distinct sites in GFP in good yields (4 mg/L, ~22% of that of wtGFP), and the mass of the purified protein sample was confirmed by MS analysis (Figure S2a and Table S1 of the Supporting Information).

To further demonstrate the utility of this system, we replaced the pEVOL-pAzF plasmid in this experiment with its counterparts encoding the aforementioned pAcF-specific or polyspecific MjTyrRS. Using pEVOL-pAcF and pUltra-Pyl^{TAA}, pAcF was incorporated into GFP at TAG3, along with eBK (4 mg/L), AlkK (0.5 mg/L), or AzK (5 mg/L) at TAA151 (Figure S2b of the Supporting Information). In each case, the full-length mutant protein was purified and verified by MS analysis (Table S1 of the Supporting Information). The combination of pEVOL (polyspecific MjTyrRS) and pUltra-Pyl^{TAA} is especially versatile, as it can be used to incorporate various combinations of UAAs that are substrates for the respective synthetases. This was demonstrated by simultaneously incorporating pAcF and AzK, pAcF and eBK, pAzF and eBK, and OMeY and eBK into TAG3 and TAA151 of GFP-3TAG-151TAA, respectively (Figure S2d of the Supporting Information). Full-length protein in each case was purified using the C-terminal hexahistidine tag (yields between 3 and 5 mg/L) and verified by MS analysis (Table S1 of the Supporting Information). The background suppression efficiency of the polyspecific MjTyrRS, in the absence of cognate UAAs, is somewhat higher than those of other engineered MjTyrRS variants. This was reflected in the control experiments for dual suppression using this plasmid, in which only the MbPylRS-specific UAA was added (Figure S2 of the Supporting Information). However, MS analyses showed homogeneous incorporation of the desired UAAs, when both were present in the expression medium (Table S1 of the Supporting Information).

Introduction of a Förster Resonance Energy Transfer (FRET) Pair by Dual Suppression. The ability to introduce two different bio-orthogonal UAAs into the same protein allows the modification of the protein with two distinct small molecules or macromolecular moieties.[12−16] Using the strategy described above, a GFP double mutant that simultaneously incorporates pAcF (TAG3) and AzK (TAA151) in good yield [5 mg/L, ~30% of the wtGFP yield (Figure 6a,b)] was expressed. To demonstrate the competence of the ketone and azide groups in this protein for site-specific conjugation reactions, we coupled the protein independently to Alexa Fluor 488 derivatized with either a hydroxylamine or a DIBO (harboring a strained cyclooctyne) moiety. The protein samples were subsequently resolved by SDS−PAGE and visualized by either UV transillumination (fluorescence) or Coomassie staining of the protein bands. Successful labeling of the ketone [using the hydroxylamine fluorophore (Figure 6c, lane 1)] and the azide groups [using the DIBO fluorophore (Figure 6c, lane 2)] was confirmed by the robust fluorescence associated with the protein bands.

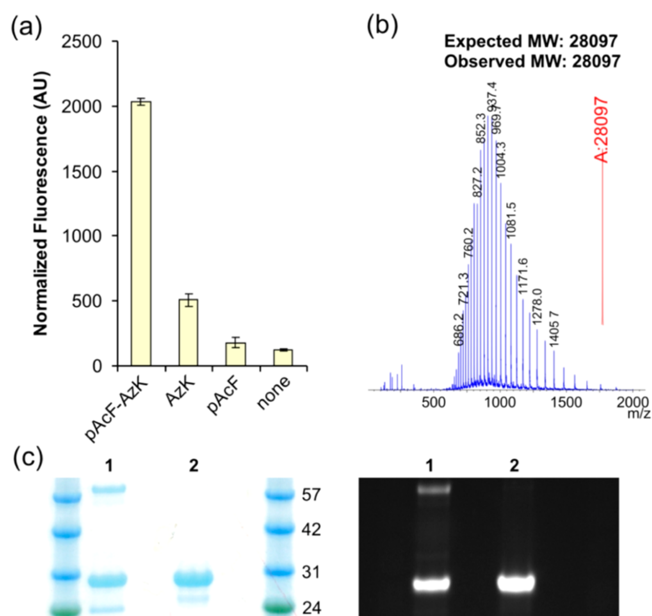

Figure 6. Incorporation and subsequent modification of two different bio-orthogonal UAAs in GFP. Full-length GFP was produced from pET-GFP-3TAG-151TAA using pEVOL-pAcF to suppress TAG and pUltra-Pyl^{TAA} to suppress TAA with pAcF and AzK, respectively. (a) Normalized fluorescence in the presence of both UAAs (pAcF and AzK), AzK alone, or pAcF alone or in the absence of both UAAs (none). (b) ESI-MS analysis of the purified protein reveals a homogeneous species with the expected molecular weight. (c) Conjugation of Alexa Fluor 488-hydroxylamine (lane 1) and Alexa Fluor 488-cyclooctyne (lane 2) to the ketone and azide groups of the resulting GFP, respectively, and their subsequent analysis by Coomassie staining (left) and UV transillumination (right), following the resolution of the samples by SDS−PAGE.

To simultaneously install two different fluorophores capable of FRET into a single protein by two bio-orthogonal conjugation reactions, a nonfluorescent protein target, ketosteroid isomerase (KSI), was used. On the basis of an available crystal structure (Protein Data Bank entry 1W00), two proximal residues in the folded form of this protein, Gln7 and His78, were chosen for the installation of the FRET pair. The double mutant (KSI-7TAA-78TAG) encoded in a pET28 expression plasmid was cotransformed with pEVOL-pAcF and pUltra-Pyl^{TAA} into the BL21(DE3) strain of *E. coli*. Expression of the mutant KSI was conducted by dual suppression, incorporating eBK or AzK at TAA7 and pAcF at TAG78. The full-length protein was purified using a C-terminal hexahistidine tag (isolated yields of 34 and 20 mg/L for eBK/pAcF and AzK/pAcF mutants, respectively) and verified by MS analysis (Figure 7a,b). To the AzK/pAcF mutants of KSI Alexa Fluor 488-hydroxylamine and Alexa Fluor 594-DIBO (containing a strained cyclooctyne) were conjugated sequentially the ketone and azide groups of pAcF and AzK, respectively. Successful conjugation was verified by SDS−PAGE analysis followed by fluorescence imaging and Coomassie staining (Figure 7c), and by MS analysis (Figure S3 of the Supporting Information). While the Alexa Fluor 488-hydroxylamine conjugation reaction went to near completion (>95%), the yield of the Alexa Fluor 594-cyclooctyne conjugation reaction was approximately 80%. The latter is due to the partial reduction of the azide to an amine during protein expression in vivo, as observed by MS analysis (Figure

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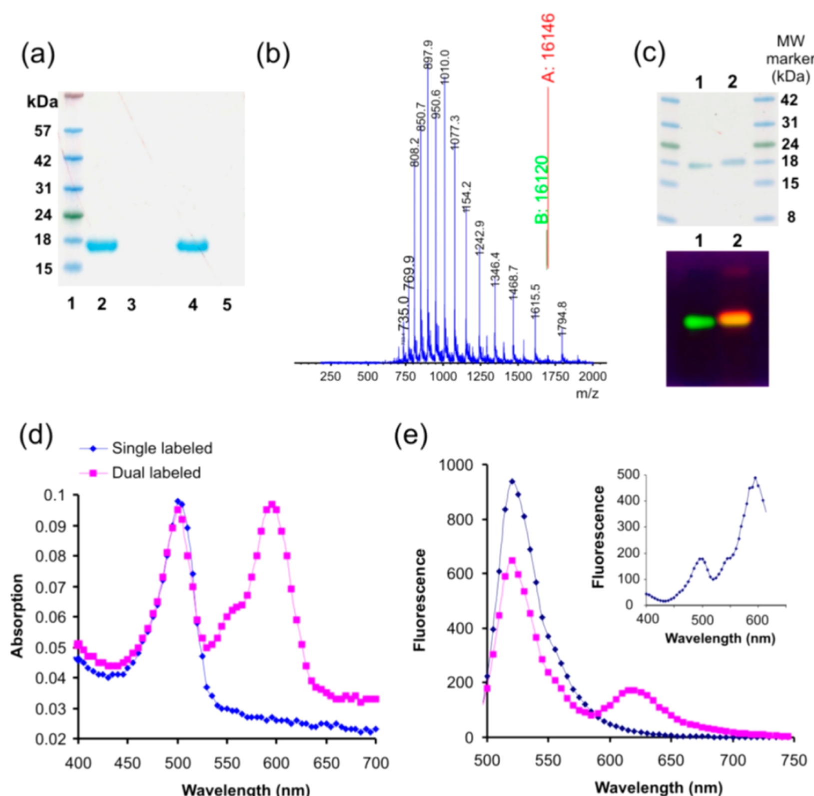

Figure 7. Incorporation and subsequent labeling of two distinct bio-orthogonal UAAs in KSI-7TAA-78TAG. (a) SDS−PAGE analysis of KSI-7TAA-78TAG expression incorporating eBK and pAcF, or AzK and pAcF: lane 1, molecular weight markers; lanes 2 and 3, expression in the presence or absence of eBK and pAcF (1 mM each), respectively; lanes 4 and 5, expression in the presence or absence of AzK and pAcF (1 mM each), respectively. (b) MS analysis of purified KSI-AzK7-pAcF78. The deconvoluted spectrum is shown in the inset and reveals the expected species (molecular weight of 16146) with a minor species (<20%), where the azide functionality of AzK is reduced to an amine (−26 molecular weight units). (c) SDS−PAGE analysis followed by Coomassie staining (top) or fluorescence imaging (bottom) of KSI-AzK7-pAcF78 labeled with Alexa Fluor 488 C_5-aminooxyacetamide alone (lane 1) or together with Alexa Fluor 594-DIBO alkyne (lane 2). Absorption (d) and fluorescence (e) (excitation wavelength of 480 nm) spectra of KSI-AzK7-pAcF78 labeled with Alexa Fluor 488 C_5-aminooxyacetamide alone (magenta) or together with Alexa Fluor 594-DIBO alkyne (blue). Shown in the inset of panel e is a plot of the fluorescence intensity of the 620 nm peak as a function of excitation wavelength, which shows absorption maxima at 590 nm (Alexa Fluor 594; self) and 490 nm (FRET from Alexa Fluor 488 fluorophore). The labeled protein concentration used for all these experiments was 25 μM.

7b and Figure S3 of the Supporting Information). FRET between the two fluorophores was verified by exciting Alexa Fluor 488 at 480 nm. While the Alexa Fluor 488 singly labeled protein had an emission maximum of 520 nm, the dual-labeled protein showed a second emission peak with a maximum at 620 nm (Figure 7e). The excitation spectrum of the latter peak revealed two absorption maxima, one consistent with the excitation of Alexa Fluor 488 and the second with excitation of Alexa Fluor 594 (Figure 7e), confirming FRET between the two fluorophores.

Generation of a Dual-Suppressor Plasmid pUltraII. The simple composition of pUltra, comprising one expression cassette each of the synthetase and tRNA, should facilitate the integration of both the tyrosyl and the pyrrolysyl suppression systems into a single suppressor plasmid. To construct such a vector, we incorporated the expression cassettes for the aaRS and the tRNA from pUltra-pAcF into pUltra-Pyl^{TAA} to generate pUltraII (Figue 8a). This plasmid encodes all of the elements of the amber-suppressing MjTyr pair and the ochre-suppressing pyrrolysine pair. To test the ability of this plasmid to conduct dual suppression, the previously described GFP-3TAG-151TAA expression plasmid was used. The suppressor and expression plasmid were cotransformed into the BL21(DE3) strain of *E. coli*, and the expression of GFP-3TAG-151TAA was attempted in TB medium using eBK and pAcF to suppress the ochre and amber codons, respectively. For comparison, the corresponding three-component system for dual suppression, using the pUltra-Pyl^{TAA} and pEVOL-pAcF suppressor plasmids, was also tested. Both systems exhibited similar levels of expression of full-length GFP [∼3−4 mg/L, 20−25% of that of wtGFP (Figure 8b)]. Thus, pUltraII provides a platform for efficient integration of multiple suppression systems to facilitate two or more UAAs to the same protein.

■ CONCLUSIONS

Here we have generated a highly efficient suppression system, consisting only of one expression cassette each for the tRNA and aaRS. This minimal suppressor system can be incorporated into vectors with different ori and antibiotic resistance markers, as demonstrated with pEVOL*tac* and pUltra, to generate highly efficient suppressor plasmids. In particular, pUltra exhibited enhanced suppression efficiency relative to the existing pEVOL vector and was especially useful when tested with weakly expressing mutants or inherently weak suppressor tRNA/aaRS pairs. The mutually compatible nature of pEVOL and pUltra was exploited to simultaneously incorporate two different UAAs into the same protein using the amber-suppressing MjTyr and the ochre-suppressing pyrrolysyl suppressor

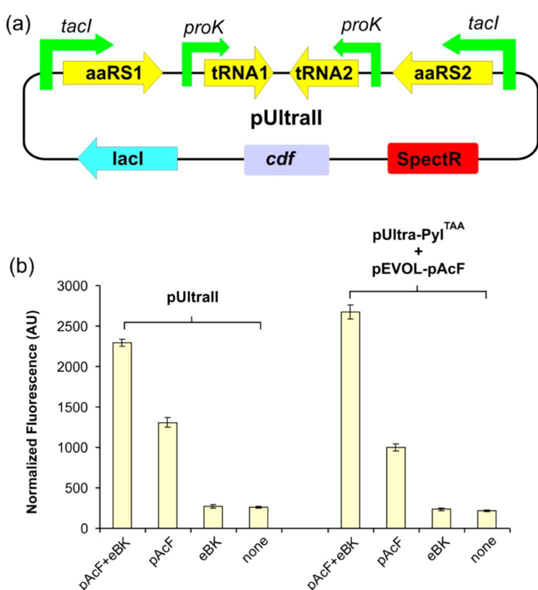

Figure 8. Construction and evaluation of pUltraII. (a) Vector map of pUltraII. aaRS1, tRNA1, aaRS2, and tRNA2 represent MbPylRS(opt), Mm-$tRNA_{UUA}^{Pyl}$ (U25C), MjTyrRS (pAcF-specific), and Mj-$tRNA_{CUA}^{Tyr}$, respectively. (b) Evaluation of the dual-suppression efficiency of pUltraII by monitoring GFP expression levels (normalized fluorescence) from pET-GFP-3TAG-151TAA in the presence of pAcF and eBK, pAcF alone, or eBK alone or in the absence of both UAAs. For comparison, the expression profile from the three-component dual-suppression system (pEVOL-pAcF, pUltra-Pyl^{TAA}, and pET-GFP-3TAG-151TAA) is also shown.

systems. Many other UAAs have been genetically encoded using the *M. jannaschii* tyrosyl- and archaeal pyrrolysyl-tRNA/aaRS pairs; the dual-suppression system described here should allow facile incorporation of various combinations of these UAAs into target proteins without the construction of complex vectors. In addition, we have optimized the suppression efficiency of the ochre suppressor pyrrolysyl pair to enhance the efficiency of dual suppression. Finally, we demonstrated that pUltra can serve as a platform for the integration of two different tRNA/aaRS suppressor pairs to generate a single plasmid capable of dual suppression. The new vector systems described herein should provide valuable new tools for facilitating single- and multiple-UAA mutagenesis in *E. coli*.

■ ASSOCIATED CONTENT

Supporting Information

Additional protein expression and characterization data, list of DNA oligomers used in this study, and sequence information for expression constructs. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Department of Chemistry and Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 N. Torrey Pines Rd., La Jolla, CA 92037. Telephone: (858) 784-9273. E-mail: schultz@scripps.edu.

Author Contributions

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Notes

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■ ABBREVIATIONS

UAA, unnatural amino acid; aaRS, aminoacyl-tRNA synthetase; ori, origin of replication; KSI, ketosteroid isomerase; TB, terrific broth.

■ REFERENCES

(1) Liu, C. C., and Schultz, P. G. (2010) Adding new chemistries to the genetic code. *Annu. Rev. Biochem. 79*, 413−444.

(2) Chen, S., Schultz, P. G., and Brock, A. (2007) An improved system for the generation and analysis of mutant proteins containing unnatural amino acids in *Saccharomyces cerevisiae*. *J. Mol. Biol. 371*, 112−122.

(3) Young, T. S., Ahmad, I., Brock, A., and Schultz, P. G. (2009) Expanding the genetic repertoire of the methylotrophic yeast *Pichia pastoris*. *Biochemistry 48*, 2643−2653.

(4) Liu, W., Brock, A., Chen, S., and Schultz, P. G. (2007) Genetic incorporation of unnatural amino acids into proteins in mammalian cells. *Nat. Methods 4*, 239−244.

(5) Sakamoto, K., Hayashi, A., Sakamoto, A., Kiga, D., Nakayama, H., Soma, A., et al. (2002) Site-specific incorporation of an unnatural amino acid into proteins in mammalian cells. *Nucleic Acids Res. 30*, 4692−4699.

(6) Wang, W., Takimoto, J. K., Louie, G. V., Baiga, T. J., Noel, J. P., Lee, K. F., et al. (2007) Genetically encoding unnatural amino acids for cellular and neuronal studies. *Nat. Neurosci. 10*, 1063−1072.

(7) Chen, P. R., Groff, D., Guo, J., Ou, W., Cellitti, S., Geierstanger, B. H., and Schultz, P. G. (2009) A facile system for encoding unnatural amino acids in mammalian cells. *Angew. Chem., Int. Ed. 48*, 4052−4055.

(8) Wang, L., Brock, A., Herberich, B., and Schultz, P. G. (2001) Expanding the genetic code of *Escherichia coli*. *Science 292*, 498−500.

(9) Ryu, Y., and Schultz, P. G. (2006) Efficient incorporation of unnatural amino acids into proteins in *Escherichia coli*. *Nat. Methods 3*, 263−265.

(10) Cellitti, S. E., Jones, D. H., et al. (2008) *In vivo* incorporation of unnatural amino acids to probe structure, dynamics, and ligand binding in a large protein by nuclear magnetic resonance spectroscopy. *J. Am. Chem. Soc. 130*, 9268−9281.

(11) Young, T. S., Ahmad, I., Yin, J. A., and Schultz, P. G. (2010) An enhanced system for unnatural amino acid mutagenesis in *E. coli*. *J. Mol. Biol. 395*, 361−374.

(12) Anderson, J. C., Wu, N., Santoro, S. W., Lakshman, V., King, D. S., and Schultz, P. G. (2004) An expanded genetic code with a functional quadruplet codon. *Proc. Natl. Acad. Sci. U.S.A. 101*, 7566−7571.

(13) Neumann, H., Wang, K., Davis, L., Garcia-Alai, M., and Chin, J. W. (2010) Encoding multiple unnatural amino acids via evolution of a quadruplet-decoding ribosome. *Nature 464*, 441−444.

(14) Wan, W., Huang, Y., Wang, Z., Russell, W. K., Pai, P. J., Russell, D. H., and Liu, W. R. (2010) A facile system for genetic incorporation of two different noncanonical amino acids into one protein in *Escherichia coli*. *Angew. Chem., Int. Ed. 49*, 3211−3214.

(15) Wu, B., Wang, Z., Huang, Y., and Liu, W. R. (2012) Catalyst-free and site-specific one-pot dual-labeling of a protein directed by two genetically incorporated noncanonical amino acids. *ChemBioChem 13*, 1405−1408.

(16) Hoesl, M. G., and Budisa, N. (2011) *In vivo* incorporation of multiple noncanonical amino acids into proteins. *Angew. Chem., Int. Ed. 50*, 2896−2902.

(17) Sun, Q. Y., Ding, L. W., He, L. L., Sun, Y. B., Shao, J. L., Luo, M., and Xu, Z. F. (2009) Culture of *Escherichia coli* in SOC medium improves the cloning efficiency of toxic protein genes. *Anal. Biochem. 394*, 144−146.
(18) Grossman, T. H., Kawasaki, E. S., Punreddy, S. R., and Osburne, M. S. (1998) Spontaneous cAMP-dependent derepression of gene expression in stationary phase plays a role in recombinant expression instability. *Gene 209*, 95−103.
(19) de Boer, H. A., Comstock, L. J., and Vasser, M. (1983) The *tac* promoter: A functional hybrid derived from the *trp* and *lac* promoters. *Proc. Natl. Acad. Sci. U.S.A. 80*, 21−25.
(20) Stuitje, A. R., Spelt, C. E., Veltkamp, E., and Nijkamp, H. J. (1981) Identification of mutations affecting replication control of plasmid Clo DF13. *Nature 290*, 264−267.
(21) Conrad, S. E., Wold, M., and Campbell, J. L. (1979) Origin and direction of DNA replication of plasmid RSF1030. *Proc. Natl. Acad. Sci. U.S.A. 76*, 736−740.
(22) Young, D. D., Young, T. S., Jahnz, M., Ahmad, I., Spraggon, G., and Schultz, P. G. (2011) An evolved aminoacyl-tRNA synthetase with atypical polysubstrate specificity. *Biochemistry 50*, 1894−900.
(23) Chatterjee, A., Xiao, H., and Schultz, P. G. (2012) Evolution of multiple, mutually orthogonal prolyl-tRNA synthetase/tRNA pairs for unnatural amino acid mutagenesis in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A. 109*, 14841−14846.
(24) Hughes, R. A., and Ellington, A. D. (2010) Rational design of an orthogonal tryptophanyl nonsense suppressor tRNA. *Nucleic Acids Res. 38*, 6813−6830.
(25) Ambrogelly, A., Gundllapalli, S., Herring, S., Polycarpo, C., Frauer, C., and Söll, D. (2007) Pyrrolysine is not hardwired for cotranslational insertion at UAG codons. *Proc. Natl. Acad. Sci. U.S.A. 104*, 3141−3146.
(26) Anderson, J. C., and Schultz, P. G. (2003) Adaptation of an orthogonal archaeal leucyl-tRNA and synthetase pair for four-base, amber, and opal suppression. *Biochemistry 42*, 9598−9608.