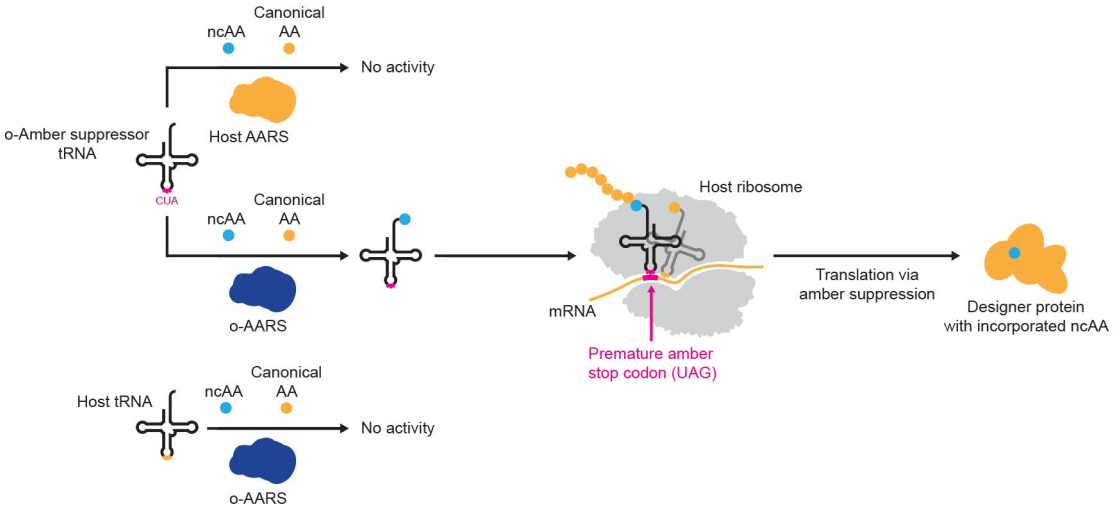
Supplementary Information

Continuous directed evolution of aminoacyl-tRNA synthetases

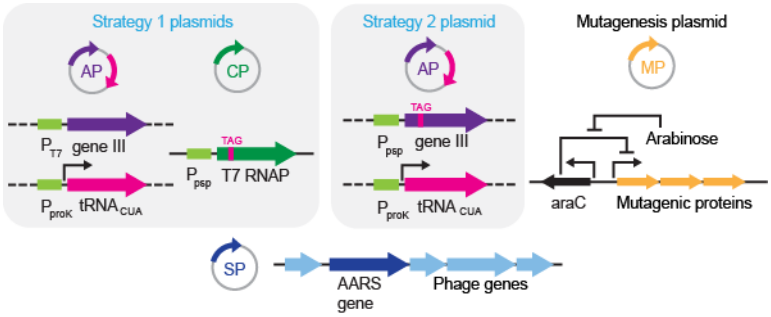
David I. Bryson[1], Chenguang Fan[2], Li-Tao Guo[3], Corwin Miller[3], Dieter Söll[3,4], & David R. Liu[1,5,6*]

[1] *Department of Chemistry and Chemical Biology, Harvard University, Cambridge, MA, 02138*
[2] *Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, AR, 72701*
[3] *Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT, 06520*
[4] *Department of Chemistry, Yale University, New Haven, CT, 06520*
[5] *Howard Hughes Medical Institute, Harvard University, Cambridge, MA, 02138*
[6] *Broad Institute of Harvard and MIT, Cambridge, MA, 02142*

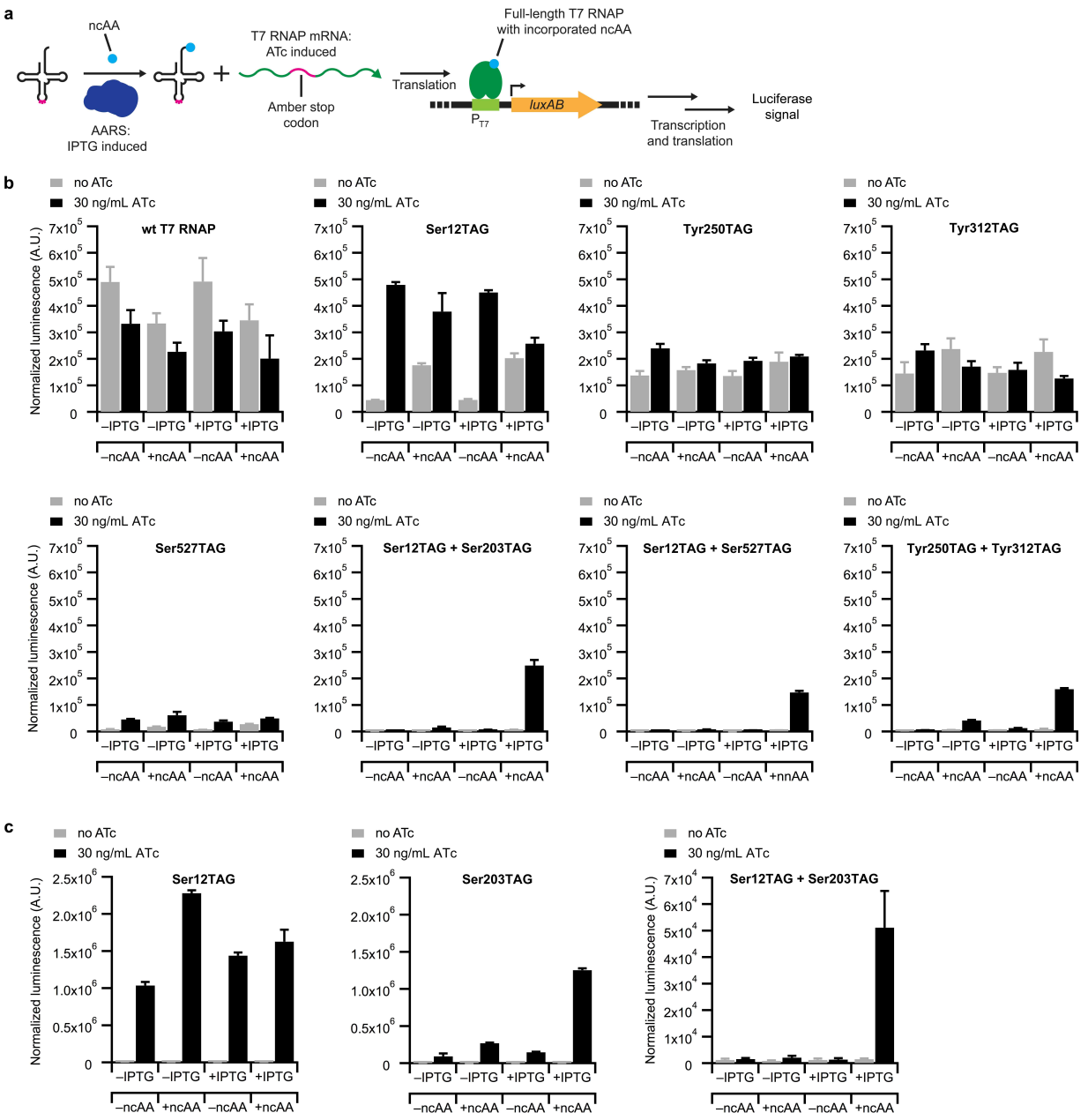
Supplementary Results



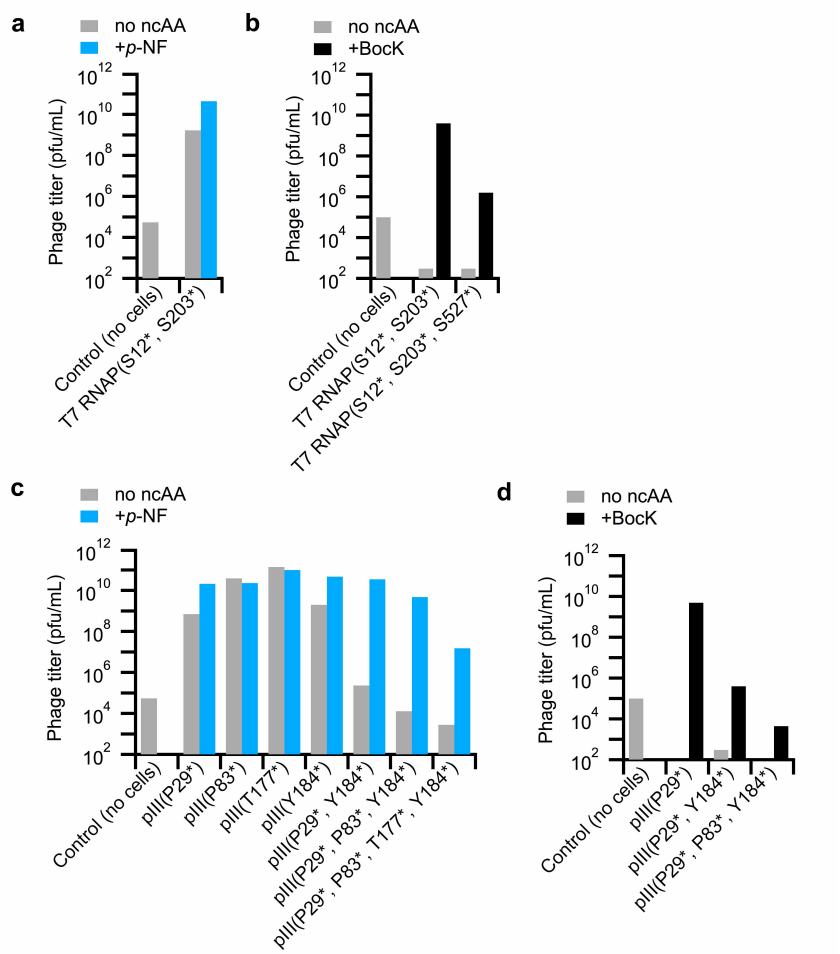
Supplementary Figure 1. Overview of orthogonal translation in biological systems. The orthogonal amber suppressor tRNA is not recognized by any of the cell's endogenous AARS enzymes, but is selectively aminoacylated by the orthogonal AARS with the desired ncAA. The charged amber suppressor tRNA decodes 'UAG' stop codons during translation of the protein of interest, enabling site-specific incorporation of the ncAA into proteins made by the cell.



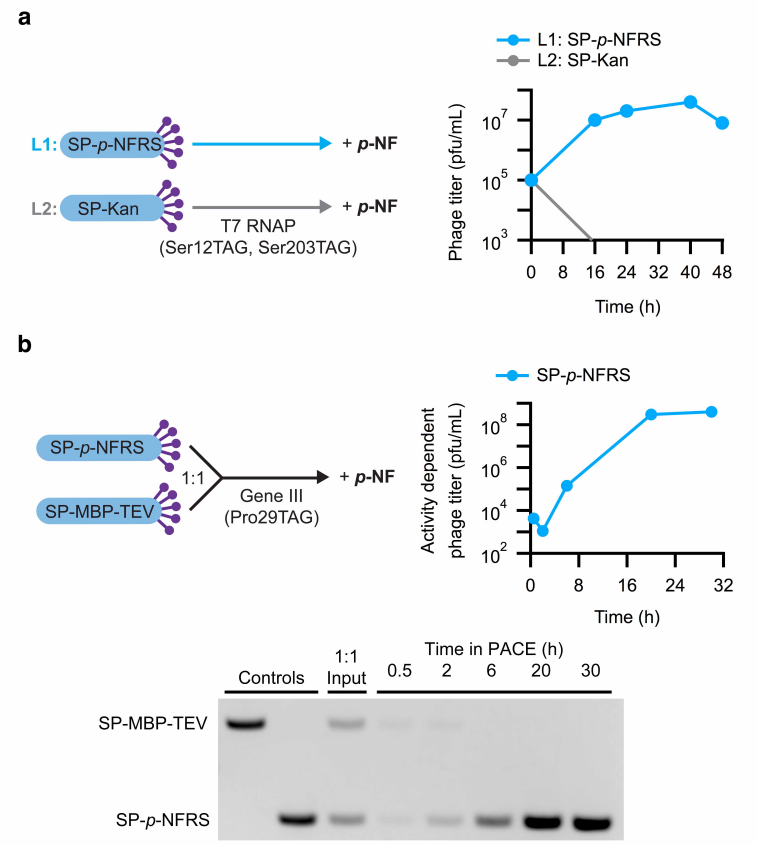
Supplementary Figure 2. Host-cell plasmids used to implement PACE positive selections for aminoacylation. The accessory plasmid (AP) in each selection strategy encodes the amber suppressor tRNA (constitutively expressed) and gene III (conditionally expressed). Strategy 1 also includes a complementary plasmid (CP) that encodes T7 RNAP. Both strategies implement a phage shock promoter (P_{psp}), which prevents expression of the downstream gene until the host is infected with the selection phage. During PACE, mutagenic proteins encoded on the mutagenesis plasmid (MP) are induced in the presence of arabinose. The selection phage (SP) encodes all phage genes except gene III, which is replaced by the evolving AARS gene.



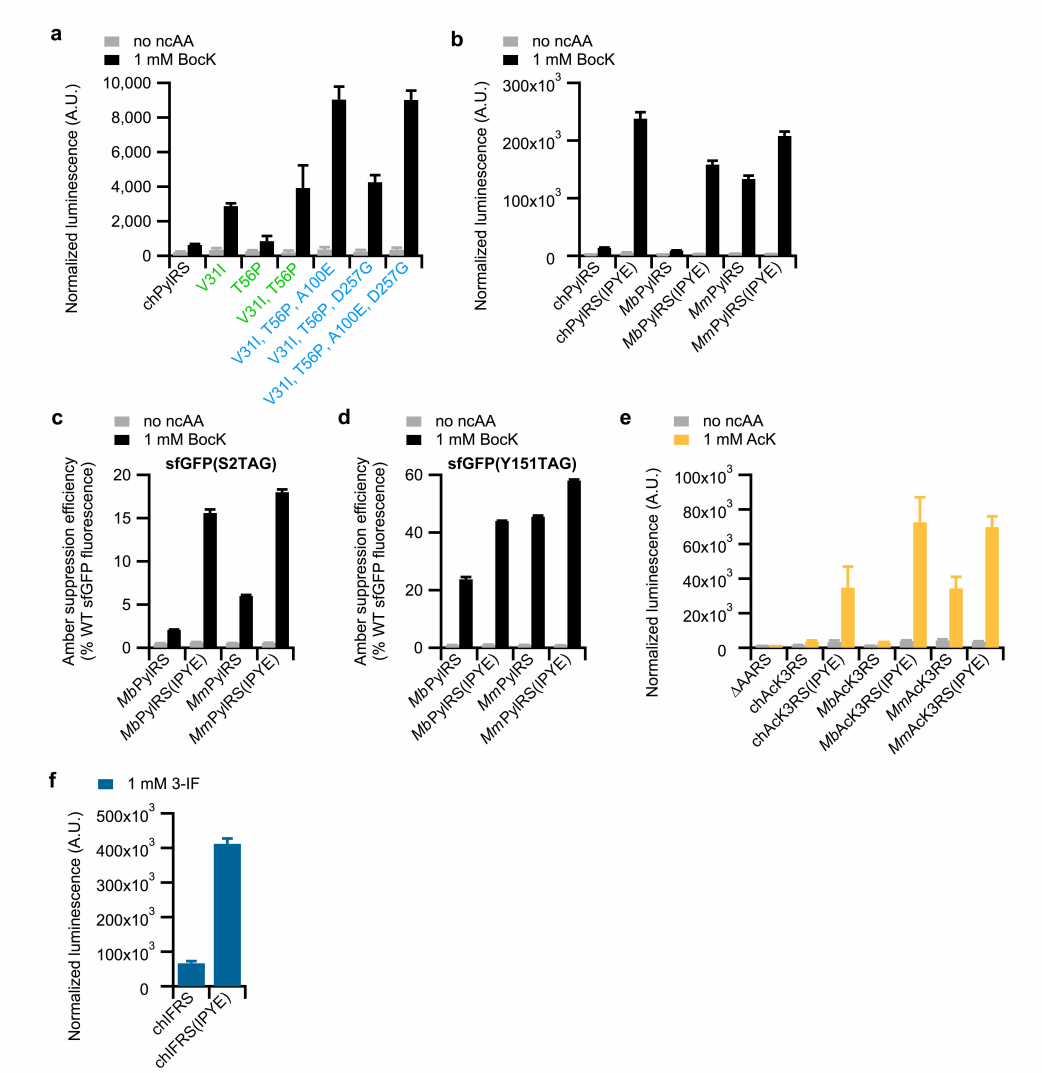
Supplementary Figure 3. Optimization of the T7 RNAP-mediated PACE positive selection for aminoacylation. Two amber stop codons in T7 RNAP are required to make reporter expression completely dependent on orthogonal translation of full-length T7 RNAP. (**a**) Luciferase reporter assay for optimizing the position and number of TAG stop codons in T7 RNAP. The orthogonal AARS (inducible with IPTG) charges the ncAA onto the amber suppressor tRNA, enabling translation of full-length T7 RNAP (inducible with anhydrotetracycline, ATc). Production of T7 RNAP results in subsequent expression of the luciferase reporter gene, *luxAB.* (**b**) Using *p*-NFRS to site-specifically incorporate *p*-NF at two positions (Ser12TAG + Ser203TAG) in T7 RNAP provided optimal reporter signal that was dependent on orthogonal AARS activity (+IPTG, +ncAA) and on expression of T7 RNAP (+ATc). (**c**) Using chPylRS, reporter signal resulting from site-specific incorporation of BocK into T7 RNAP(Ser12TAG + Ser203TAG) suggests broad ncAA-tolerance at both sites of ncAA installation. Each value and error bar in **b** and **c** reflects the mean and s.d. of at least three independent biological replicates.



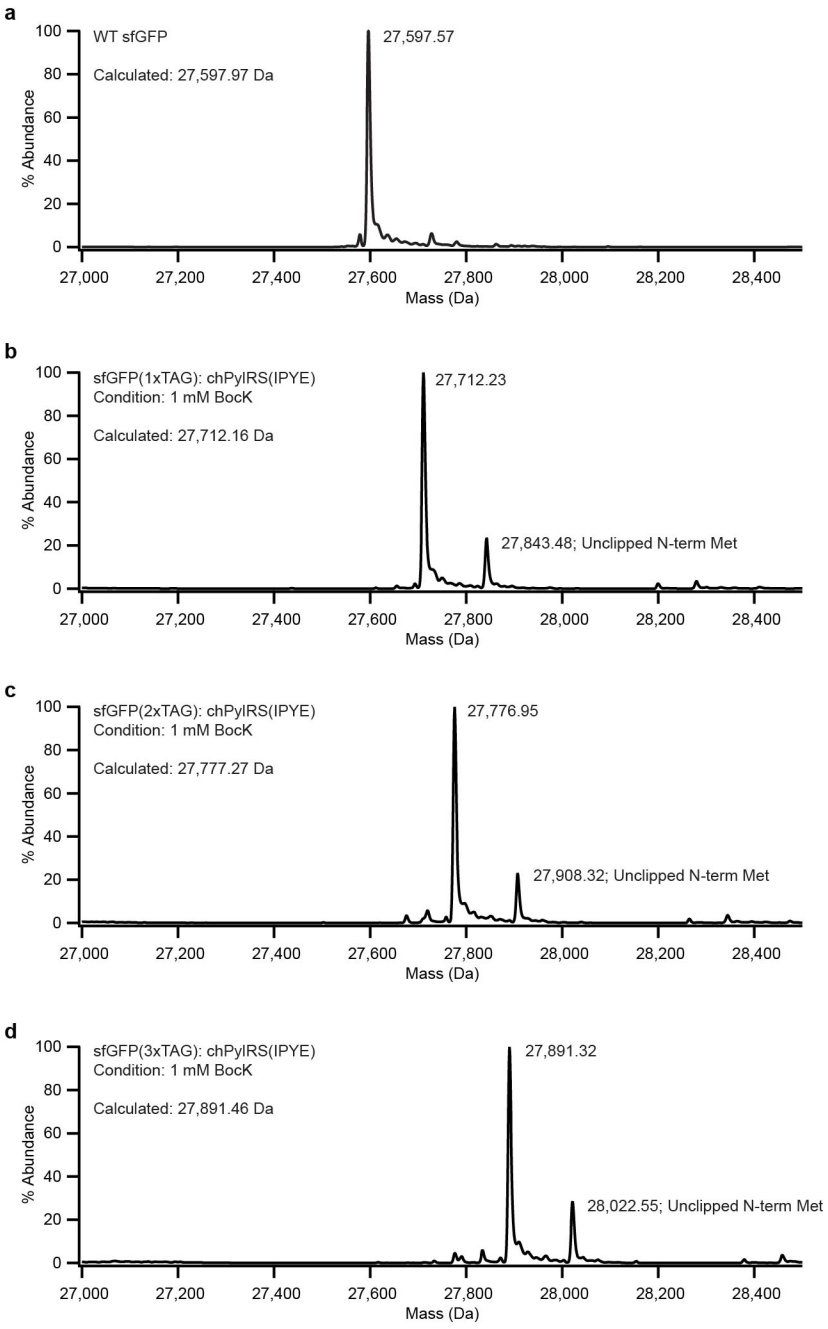
Supplementary Figure 4. Non-continuous propagation of SP in positive selections designed for PACE. To confirm activity dependence of phage propagation for each of the two positive selections (suppression of stop codons in T7 RNAP or in gene III), phage titers resulting from 16 h of propagation in batch culture were compared for SP expressing *p*-NFRS (**a, c**) or chPylRS (**b, d**). In each experiment, equal amounts of SP encoding the AARS of interest were used to infect cultures of S1030 host cells harboring the required PACE AP and CP plasmids in the presence or absence of the ncAA. Controls representing the starting titers for each set of experiments were prepared by diluting the same amount of SP into media lacking cells. Results indicate that selection stringency increases as the number of stop codons is increased in T7 RNAP (**a, b**) or gIII (**c, d**).



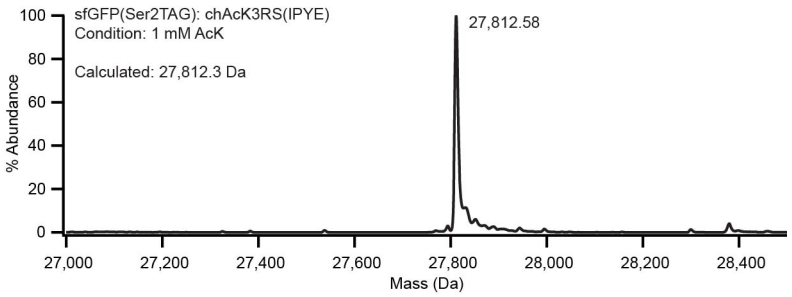
Supplementary Figure 5. Positive selections for aminoacylation support activity-dependent, continuous propagation in PACE. (**a**) In lagoon 1 (L1) supplemented with 1 mM *p*-NF, SP-*p*-NFRS propagates for 48 h of PACE using the selection based on amber suppression of two stop codons in T7 RNAP. SP-Kan, which lacks AARS activity, however, rapidly washed out of lagoon 2 (L2) by the first time point (16 h) under identical conditions. (**b**) Phage were propagated for 30 h of PACE in the presence of 1 mM *p*-NF starting from a 1:1 mixture of SP-*p*-NFRS and SP-MBP-TEV using the selection based on amber suppression of a single stop codon in gene III. Activity-dependent phage titers and PCR analysis of phage taken from each time point sampled during PACE confirmed that SP-*p*-NFRS propagated exclusively while SP-MBP-TEV rapidly washed out.



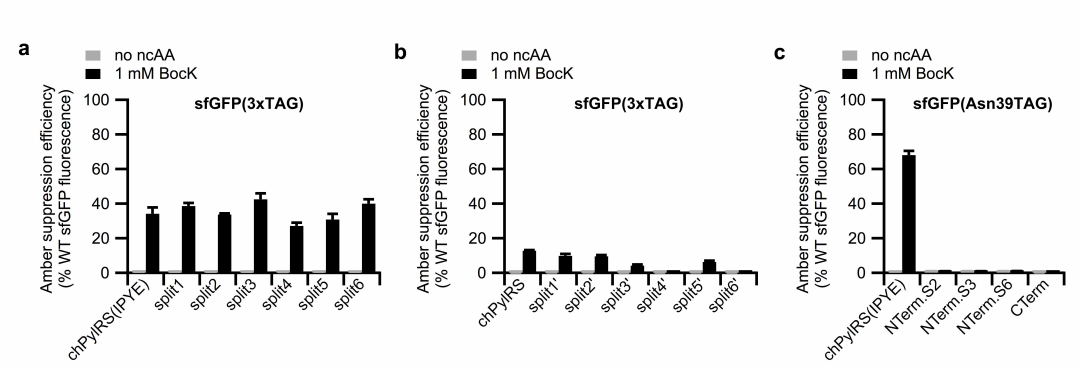
Supplementary Figure 6. Mutations emerging from PACE enhance the activity of PylRS variants on their target ncAA. (**a**) Contributions toward improved activity from consensus mutations in chPylRS generated during PACE segments Pyl-1 (green) and Pyl-2 (cyan). (**b**, **c**, **d**) Transplantation of the activity-enhancing PACE mutations V31I, T56P, H62Y, and A100E (IPYE) into *M. barkeri* (*Mb*) or *M. Mazei* (*Mm*) PylRS greatly improved the expression levels of luciferase containing the ncAA BocK at position 361 (**b**) and the expression levels of sfGFP containing a BocK at position 2 (**c**) or position 151 (**d**). (**e**, **f**) Transplantation of the 'IPYE' mutations into multiple variants of AcK3RS (**e**) or into the chimeric IFRS (**f**) improved expression of luciferase containing the ncAA residue at position 361. Each value and error bar in **b**-**e** reflects the mean and s.d. of at least three independent biological replicates.



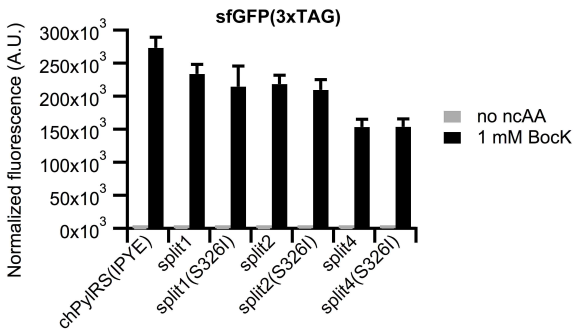
Supplementary Figure 7. ESI-MS analysis of purified sfGFP containing up to three BocK residues produced by chPylRS(IPYE). Analysis of purified wild type sfGFP (**a**) or sfGFP containing one (**b**), two (**c**) or three (**d**) BocK residues produced by chPylRS(IPYE) in the presence of 1 mM ncAA. BocK substitutions in sfGFP were made in response to premature amber stop codons at positions 39 (1xTAG), 39 and 151 (2xTAG), or 39, 135, and 151 (3xTAG). Protein was expressed in TOP10 cells in LB media. The major peak in each of the spectra was in agreement with the calculated mass of BocK incorporation. In each of the spectra containing BocK, a minor peak corresponding to an unclipped N-terminal methionine was also observed (calculated mass + 131.19 Da).



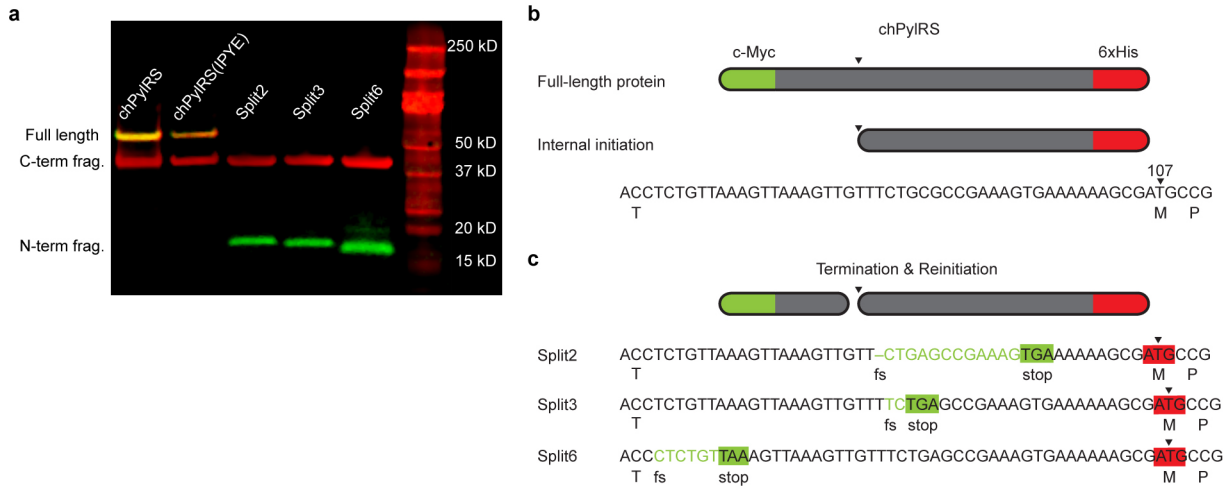
Supplementary Figure 8. ESI-MS analysis of purified sfGFP containing an AcK residue at position 2 produced by chAcK3RS(IPYE) in the presence of 1 mM AcK. Protein was expressed in TOP10 cells in LB media, and the major peak found at 27,812.58 Da was in agreement with the calculated value (27,812.3 Da).



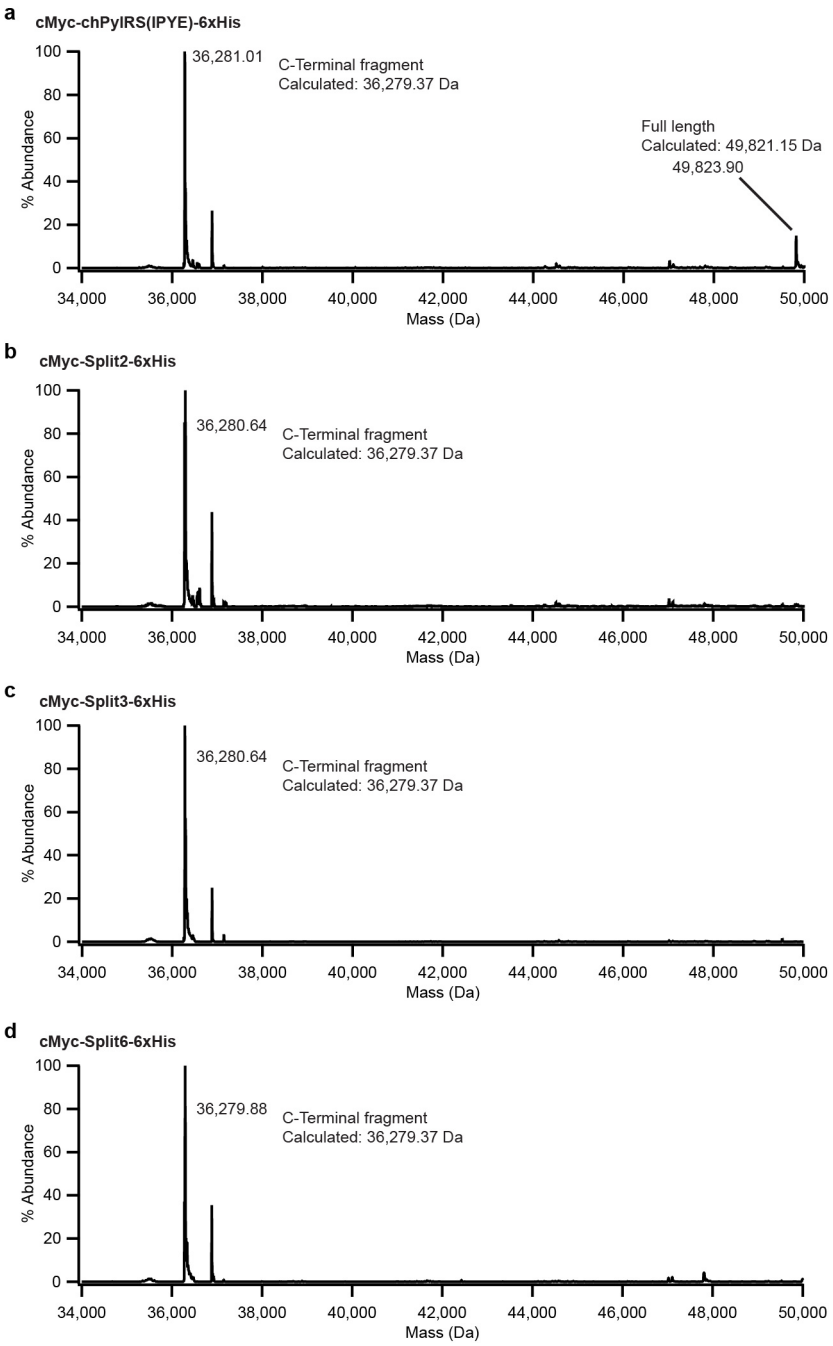
Supplementary Figure 9. Characterization of split variants of chPylRS emerging from PACE. Evolved split variants of chPylRS require the 'IPYE' tetramutation to retain high activity. Aminoacylation is dependent on both the N- and C-terminal fragments of the chPylRS variants shown. (**a, b**) The relative expression sfGFP containing three premature stop codons at positions 39, 135, and 151 (sfGFP(3xTAG)) was compared in the presence or absence of 1 mM BocK for the six, split proteins containing the 'IPYE' tetramutation (**a**) or with variants lacking the tetramutation (**b**). (**c**) The relative expression of sfGFP(Asn39TAG) in the presence of the unsplit chPylRS(IPYE) was compared to expression in the presence of the N-terminal fragments of split2 (NTerm.S2), split3 (NTerm.S3), or split6 (NTerm.S6) or the C-terminal fragment (CTerm) that would result from reinitiation at Met-107. Each value and error bar reflects the mean and s.d. of four independent biological replicates.



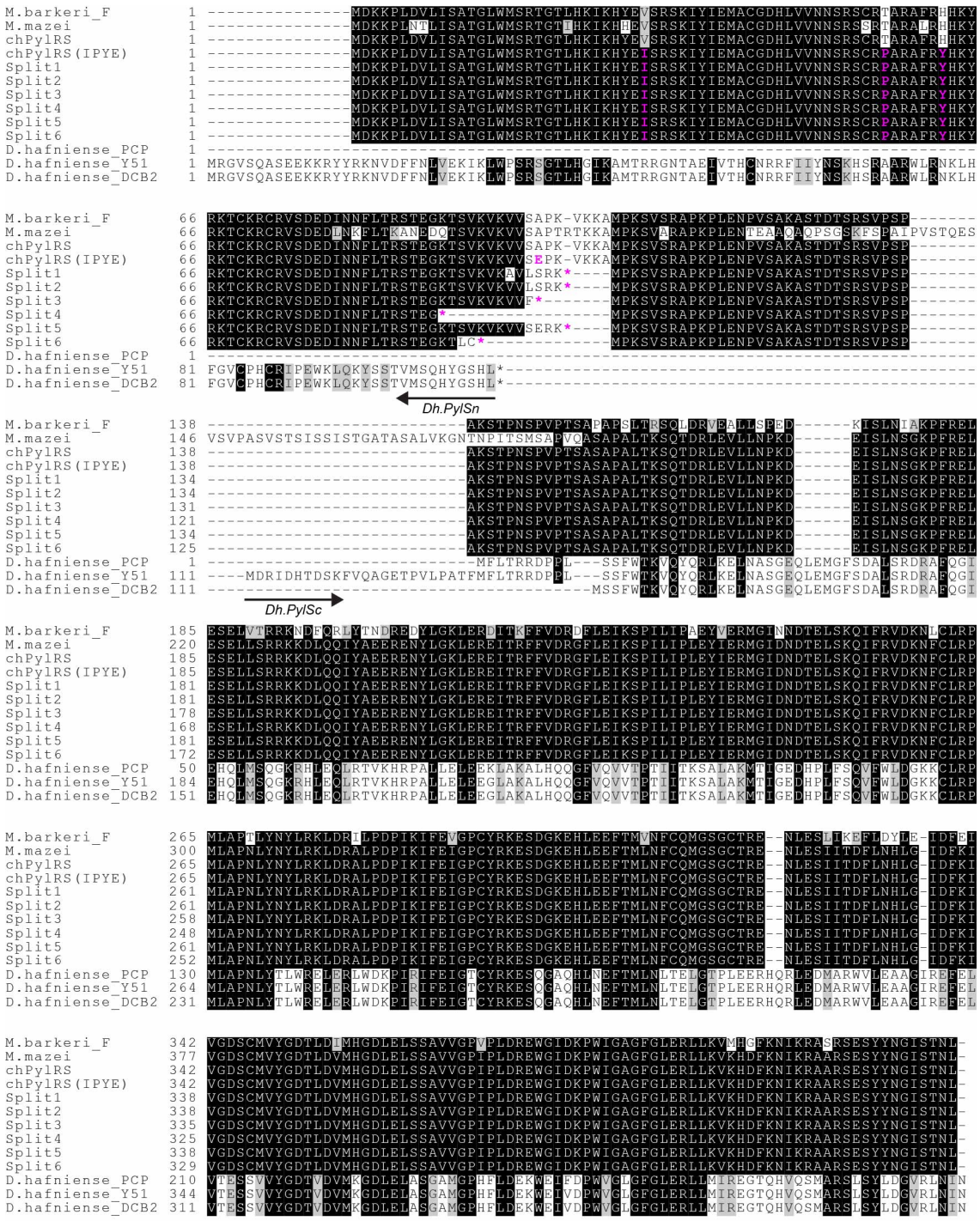
Supplementary Figure 10. Characterization of the S326I mutation emerging from lagoon 2 during the Pyl-3 segment of PACE. The relative activity of split1, split2, and split3 containing the additional mutation, S326I, were compared to variants lacking the mutation and to the full-length chPylRS(IPYE). Each variant was used to produce sfGFP(3xTAG) containing three premature stop codons at positions 39, 135, and 151. Each value and error bar represents the s.d. of four independent biological replicates.



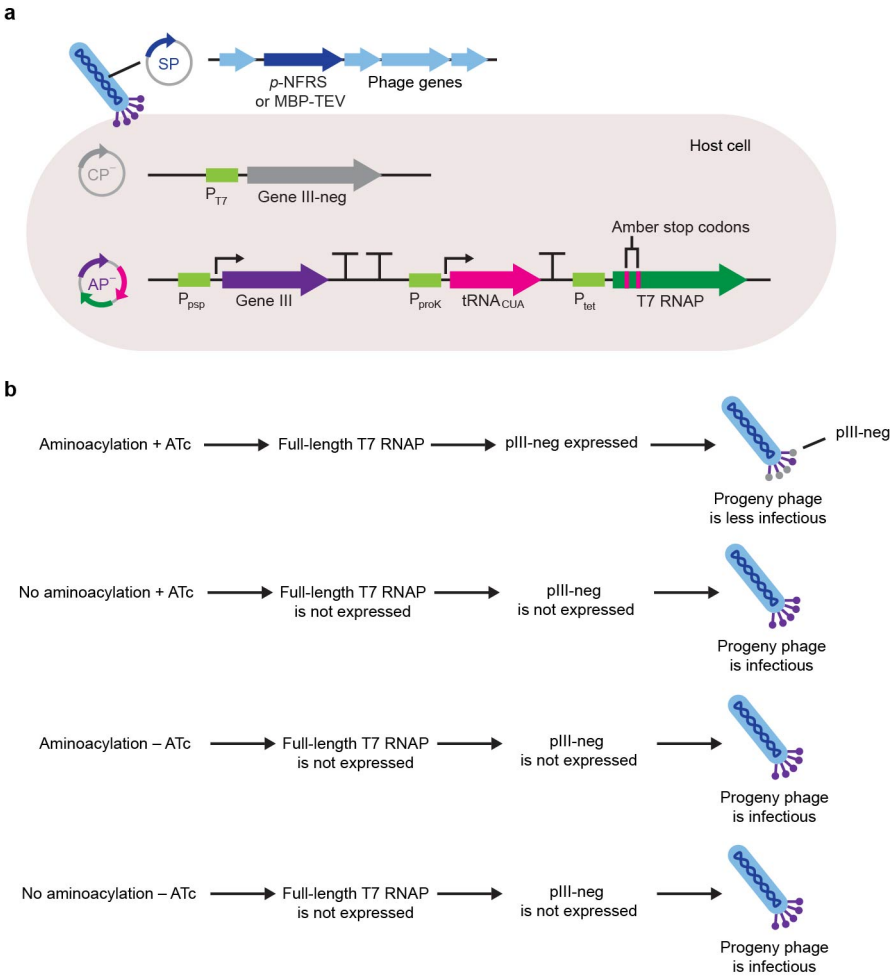
Supplementary Figure 11. Western blot analysis of full-length and split chPylRS variants from PACE. (**a, b, c**) The chPylRS variants were N-terminally fused to a c-Myc tag and C-terminally fused to a 6xHis tag to enable two-color detection of the proteins expressed in BL21 star DE3 cells. Western blot analysis of the cell lysates (**a**) indicated that chPylRS and chPylRS(IPYE) were expressed in two forms in a 1:1 ratio, either as a full-length protein with the N- and C-termini intact, or as a truncated C-terminal fragment. The absence of a corresponding N-terminal fragment from these variants suggests that the C-terminal fragment results from an internal start site in the gene at Met-107 (**b**), rather than as the result of hydrolysis. Each of the split variants (split2, split3, and split 6) are expressed as two, distinct N- and C-terminal fragments indicating termination of translation at the premature stop codon due to a frameshift (fs) mutation and reinitiation at an internal start site (**c**).



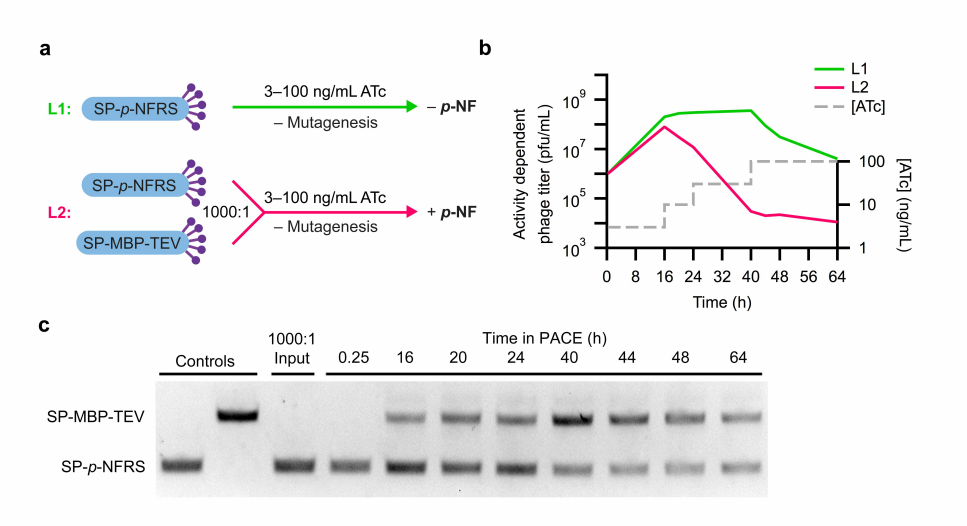
Supplementary Figure 12. ESI-MS analysis of affinity-tagged Ni-NTA-purified chPylRS variants from PACE. The evolved synthetases, chPylRS(IPYE) (**a**), Spit2 (**b**), Split3 (**c**), and Split6 (**d**) were labeled with an N-terminal c-Myc-tag and a C-terminal 6xHis-tag and purified over Ni-NTA resin prior to ESI-MS analysis. In the split variants of chPylRS, the N-terminal fragment is lost upon affinity purification. Protein was expressed in BL21 star DE3 cells in LB media. The major peaks in each spectra were in agreement with the calculated mass of the full-length enzyme, chPylRS(IPYE) (**a**), or the C-terminal fragment resulting from reinitiation at position Met-107 (**a-d**).



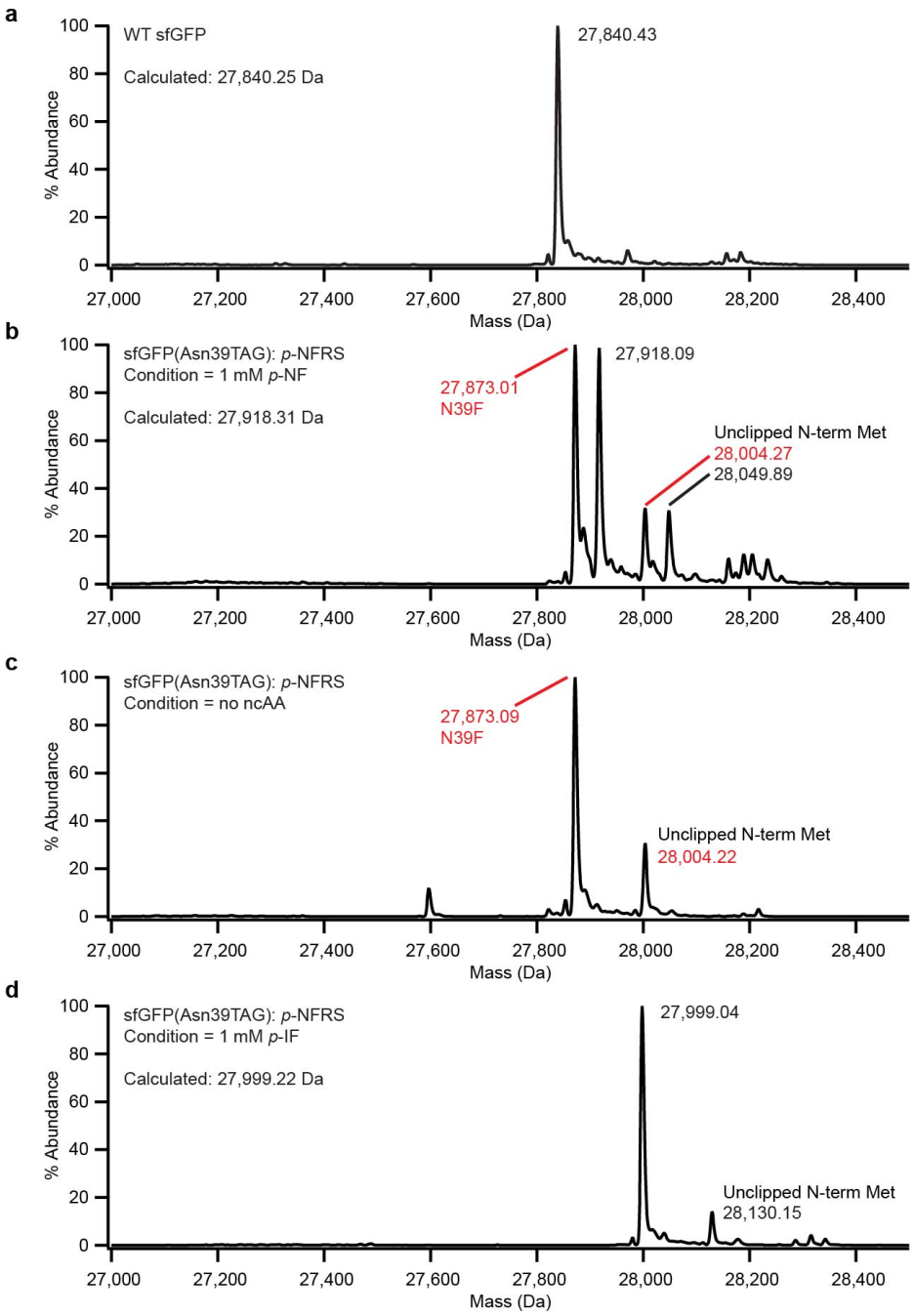
Supplementary Figure 13. Alignment of PylRS sequences from multiple organisms and from PACE variants. Activity enhancing mutations from PACE and premature stop codons (∗) that emerged in each of the split variants are shown in magenta. Note that the activity-enhancing A100E mutation became A100S in Split1 and Split 2 due to the frameshift. Split3, Split4, and Split6 each lack the A100E mutations because they terminate earlier in the sequence. Arrows denote the *PylSn* and *PylSc* gene products of the *D. hafniense* strains. The *PylSn* from the *D. hafniense* strain PCP-1 has not been sequenced, and is not included in the figure.



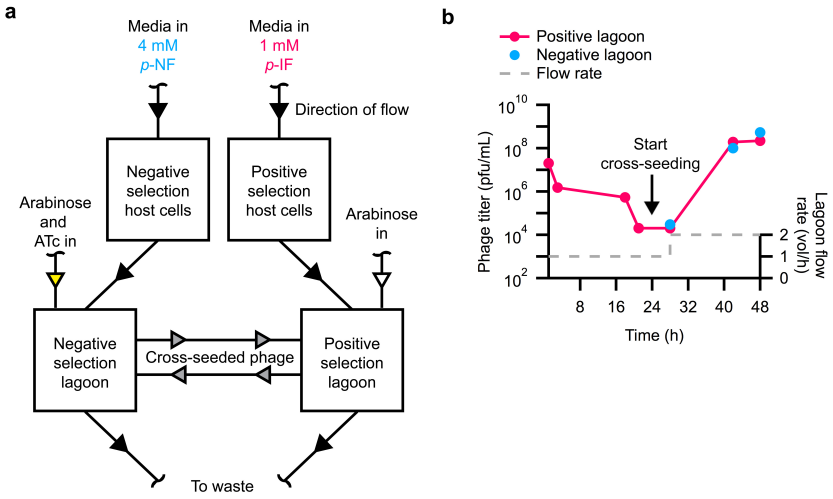
Supplementary Figure 14. Overview of the PACE negative selection for AARS activity using the dominant-negative variant of pIII (pIII-neg). (**a**) Diagram of PACE negative selection plasmids. PACE host cells (S1030) are cotransformed with the negative-selection accessory plasmid (AP^-) and a negative-selection complementary plasmid (CP^-). When an SP infects the negative selection host, production of pIII protein from gene III is induced from the phage shock promoter (P_{psp}) of the AP^-. If the AARS encoded by the SP can catalyze aminoacylation under the conditions of the negative selection (e.g., in the absence of ncAA), full-length T7 RNAP is produced from the AP^- through suppression of amber stop codons at position 12 and 203 of the T7 RNAP gene. When full-length T7 RNAP is produced, expression of gene III-neg is induced from the T7 promoter (P_{T7}) of the CP^- resulting in production of the dominant-negative pIII-neg protein. The infectivity of progeny phage decreases with the amount of pIII-neg in the host cell. Expression levels of the T7 RNAP gene on the AP^- are also controlled by an ATc-inducible promoter (P_{tet}), allowing the negative selection to be turned on or off during PACE. (**b**) Diagram of inputs and outputs of the AND logic gate created by the PACE negative selection. The dominant-negative pIII-neg protein is produced only in the presence of both aminoacylation activity and ATc. In the absence of either negative-selection input, progeny phage are infectious and carry forward the encoded AARS into the subsequent round of evolution in PACE.



Supplementary Figure 15. Validation of the PACE negative selection. (**a**) Mock PACE experiments were performed in parallel to demonstrate that the negative selection is dependent on both aminoacylation activity and the concentration of ATc. In lagoon 1 (L1), SP-*p*-NFRS was propagated in the absence of substrate amino acid (–*p*-NF) to determine the maximum concentration of ATc that could be tolerated without decreasing the rate of phage propagation when aminoacylation does not occur. In lagoon 2 (L2), SP-*p*-NFRS and SP-MBP-TEV were both propagated in the presence of the *p*-NFRS substrate (+*p*-NF) to determine the minimum concentration of ATc that would support negative selection when aminoacylation does occur. (**b**) Activity-dependent titers were measured to detect the relative amount of active SP-*p*-NFRS present in the lagoons at each sampled time point of PACE. In L1 (green line), the maximum concentration of ATc (broken gray line) that did not affect phage propagation was 30 ng/mL. In L2 (magenta line), the minimum concentration of ATc that induced negative selection against aminoacylation was 10 ng/mL. (**c**) PCR analysis of phage from each sampled time point of L2 confirms that the inactive SP-MBP-TEV was selectively enriched from a 1000:1 excess of SP-*p*-NFRS at time points that correspond to ATc concentrations between 10 and 30 ng/mL (16–40 h of PACE).

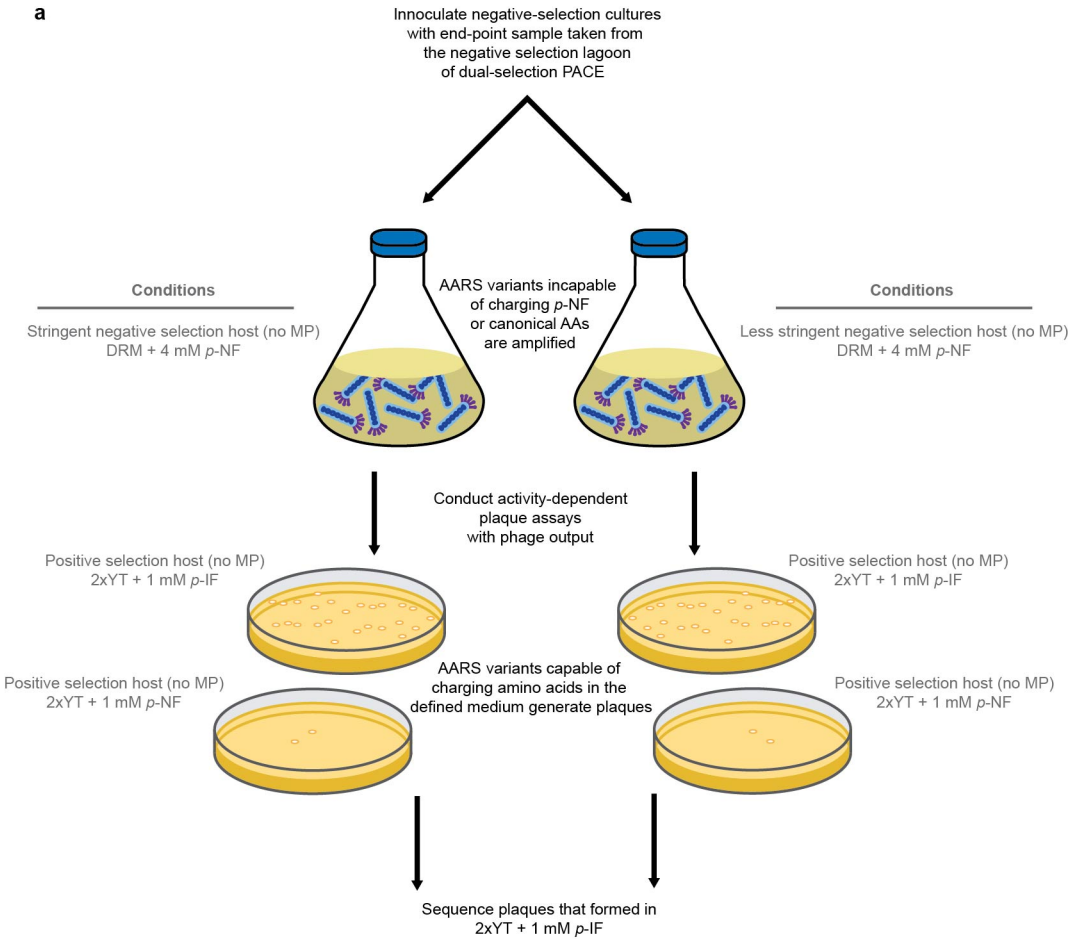


Supplementary Figure 16. The previously evolved AARS, *p*-NFRS, accepts multiple amino acid substrates. ESI-MS analysis of purified wild type sfGFP (**a**) or sfGFP(Asn39TAG) expressed with *p*-NFRS in the presence of 1 mM *p*-NF (**b**), no ncAA (**c**), or 1 mM *p*-IF (**d**) demonstrates that *p*-NFRS accepts Phe, *p*-NF, and *p*-IF. Protein was expressed in BL21 star DE3 cells in LB media. (**b**) A peak corresponding to incorporation of *p*-NF into sfGFP was observed at 27,918.09 Da (calculated: 27,918.31 Da). (**b, c**) Peaks corresponding to incorporation of Phe (red) were found at 27,873.01 Da and 27873.09 Da, respectively, (calculated: 27,873.32 Da) from expression in the presence or absence of 1 mM *p*-NF. (**c**) A peak corresponding to incorporation of *p*-IF into sfGFP was found at 27,999.04 Da (calculated: 27,999.22 Da). Minor peaks in each spectrum correspond to an unclipped N-terminal methionine (calculated mass +131.19 Da).



Supplementary Figure 17. Dual-selection PACE of the polyspecific *Mj*TyrRS variant, *p*-NFRS, to evolve selective activity on *p*-IF. (**a**) Diagram of chemostats and lagoons during dual-selection PACE. DRM media supplemented with 4 mM *p*-NF was pumped into the negative selection lagoon and DRM media supplemented with 1 mM *p*-IF was pumped into the positive selection lagoon. Host cell cultures from each chemostat were pumped through the corresponding lagoons that were supplemented with required inducers (ATc and arabinose). The opposing lagoons were coupled such that material was continuously exchanged ('cross-seeded') between each lagoon at a 50-fold slower flow rate (gray arrows) with respect to the flow rate from the chemostats through each lagoon (black arrows). (**b**) Plot of phage titers measured from samples taken at the indicated time points from each lagoon during PACE. Positive selection was conducted exclusively for the first 24 h of the experiment, and dual-selection began at the 24-h time point by cross-seeding phage between the opposing lagoons. The flow rate from the chemostats through the lagoons (broken gray line) was doubled after the two lagoons were coupled, and the flow rate of cross-seeded material was adjusted to maintain 50-fold dilution into the opposing selections.

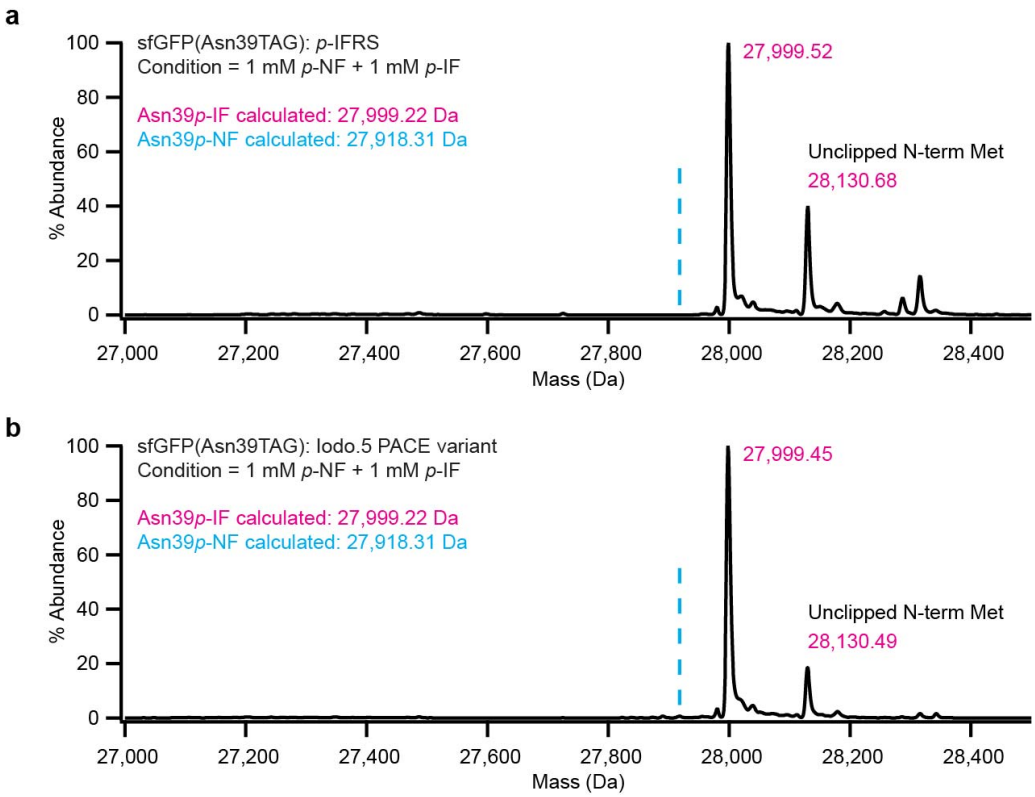
a



b

Non-continuous negative selection	Enrichment Factor (pfu in *p*-IF / pfu in *p*-NF)	Clonal phage isolate	Mutations
Stringent	267	Iodo.1	S107P
		Iodo.2	RBS mutation
		Iodo.3	RBS mutation
		Iodo.4	RBS mutation
Less stringent	233	Iodo.5	L69F, V235I
		Iodo.6	RBS mutation
		Iodo.7	G163C, N211K
		Iodo.8	S207A

Supplementary Figure 18. Non-continuous counterselections to isolate *p*-IF-selective evolved AARS variants after dual-selection PACE. (**a**) Two counterselections were performed in parallel without enhanced mutagenesis (no MP) on the evolved pool of SP sampled from the negative-selection lagoon at the end of dual-selection PACE. Negative selections were performed in batch culture to non-continuously propagate phage lacking unwanted AARS activity on canonical amino acids and *p*-NF. The stringent negative selection (left) was performed in host cells containing an AP^{-}:CP^{-} pair in which the ATc-inducible promoter driving expression of T7 RNAP(Ser12TAG, Ser203TAG) on AP^{-} (**Supplementary Fig 13a**) was replaced with the strong, P_{proD} constitutive-promoter[1]. A less stringent negative selection was performed (right) using an AP^{-}:CP^{-} pair in which the weaker P_{proA} constitutive-promoter[1] was upstream of T7 RNAP(Ser12TAG, Ser203TAG). SPs that propagated overnight in the non-continuous negative selection were isolated and used to infect positive-selection host cells to conduct activity-dependent plaque assays in the presence of *p*-NF or *p*-IF. Plaques that formed in the presence of the desired amino acid, *p*-IF, were isolated and subjected to DNA sequencing. (**b**) Results from parallel counterselections. The enrichment factor reports the number of activity-dependent plaques that formed in 1 mM *p*-IF divided by the number of plaques that formed in 1 mM *p*-NF. Mutations shown in red indicate that the ribosome-binding site (RBS) driving translation of the AARS was mutated; these clones were not further characterized.



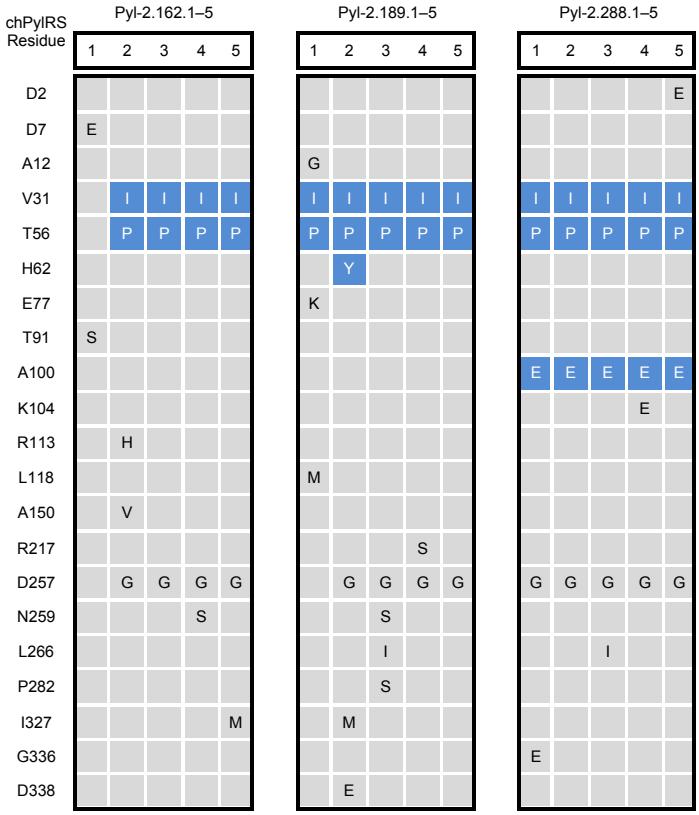
Supplementary Figure 19. The PACE-evolved Iodo.5 variant is highly selective for the desired substrate, p-IF. ESI-MS analysis of purified sfGFP from expression of sfGFP(Asn39TAG) with *p*-IFRS (**a**) or Iodo.5 (**b**) in LB media supplemented with both 1 mM *p*-NF and 1 mM *p*-IF demonstrates that each AARS enzyme selectively incorporates *p*-IF. (**a, b**) A peak corresponding to incorporation of *p*-IF into sfGFP was found at 27,999.52 Da and 27,999.45 Da, respectively (calculated: 27,999.22 Da). Incorporation of *p*-NF into sfGFP was calculated to have a mass of 27,918.31 Da (dashed blue line).

Supplementary Table 1. Summary of mutations observed in PACE segment Pyl-1

chPylRS Residue	Pyl-1.120.1–8							
	1	2	3	4	5	6	7	8
D7			A					
V31	I	I	I	I	F	I	I	I
41A						E		
T56	P	P	P	P			P	P
A100		T						
S127	P			P				
A152	V							
D257		G						
G343		D						

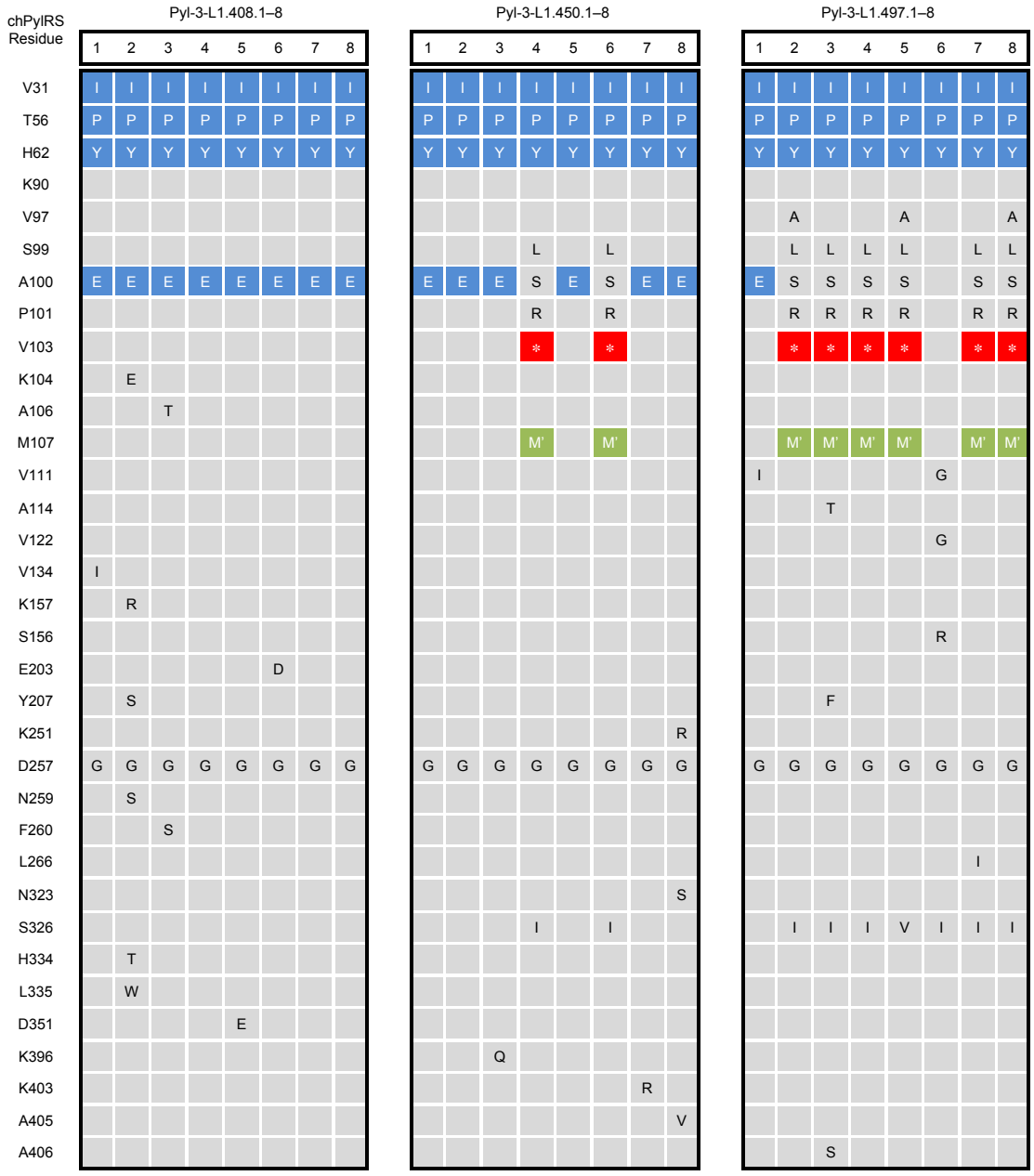
Mutations in chPylRS from the Pyl-1 segment were determined by Sanger sequencing of eight clonal SP isolates from 120 h of total PACE. Only coding mutations are shown. Mutations highlighted in blue were shown to be responsible for enhancing the activity of chPylRS.

Supplementary Table 2. Summary of mutations observed in PACE segment Pyl-2



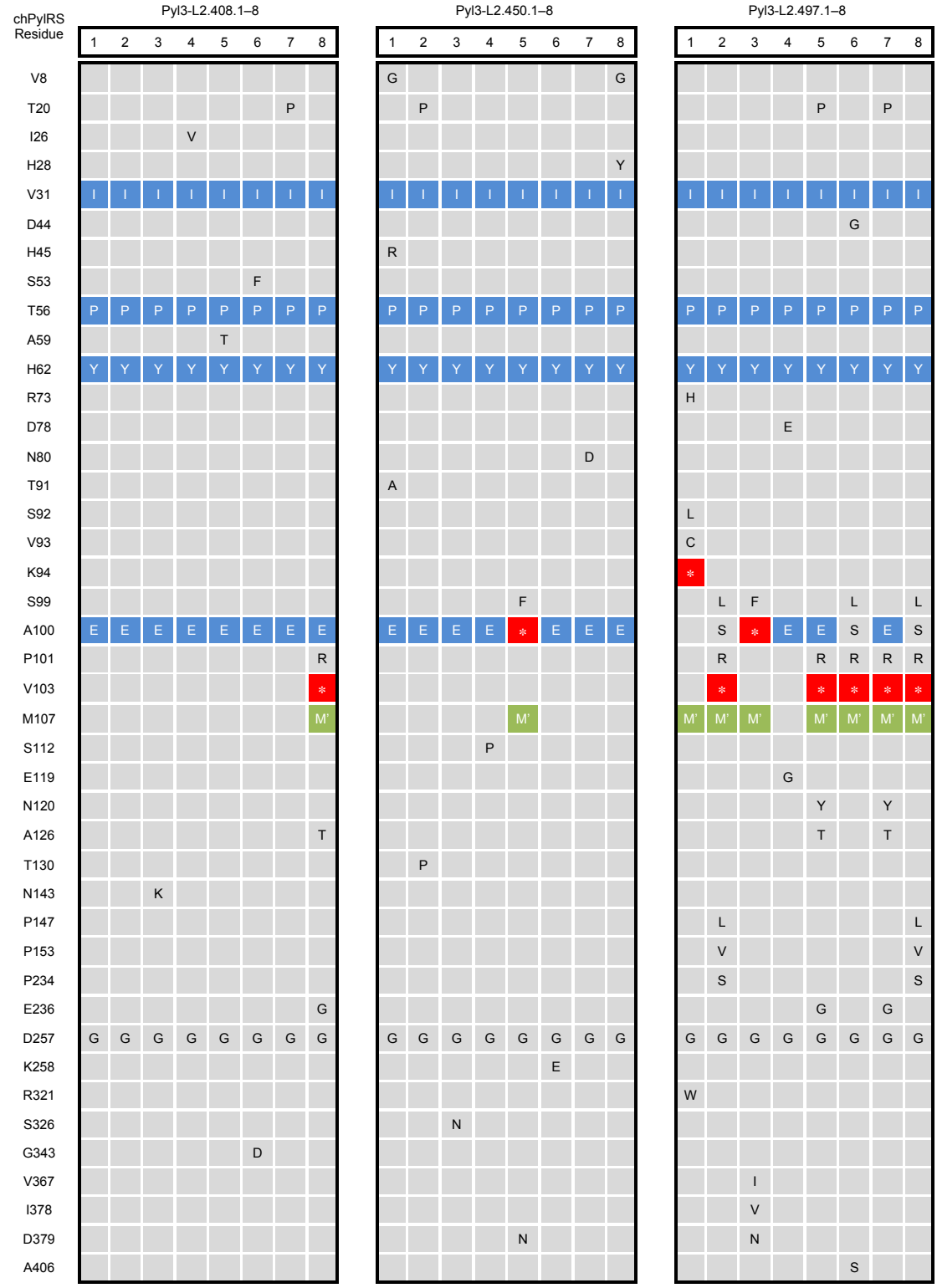
Mutations in chPylRS from the Pyl-2 segment were determined by Sanger sequencing of five clonal SP isolates from 162 h, 189 h, and 288 h of total PACE. Only coding mutations are shown. Mutations highlighted in blue were shown to be responsible for enhancing the activity of chPylRS.

Supplementary Table 3. Summary of mutations observed in lagoon 1 of PACE segment Pyl-3



Mutations in chPylRS from lagoon 1 (L1) of the Pyl-3 segment were determined by Sanger sequencing of eight clonal SP isolates from 408 h, 450 h, and 497 h of total PACE. Only coding mutations are shown. Mutations highlighted in blue were shown to be responsible for enhancing the activity of chPylRS. Mutations highlighted in red indicate stop codons that resulted in split-protein variants in which translation reinitiates at the position corresponding to Met-107 of chPylRS (M', highlighted in green).

Supplementary Table 4. Summary of mutations observed in lagoon 2 of PACE segment Pyl-3



Mutations in chPylRS from lagoon 2 (L2) of the Pyl-3 segment were determined by Sanger sequencing of eight clonal SP isolates from 408 h, 450 h, and 497 h of total PACE. Only coding mutations are shown. Mutations highlighted in blue were shown to be responsible for enhancing the activity of chPylRS. Mutations highlighted in red indicate stop codons that resulted in split-protein variants in which translation reinitiates at the position corresponding to Met-107 of chPylRS (M', highlighted in green).

Supplementary Table 5. Kinetic parameters of chPylRS variants using L-pyrrolysine substrate

PylRS variant	k_{cat}, s^{-1} x 10^{-3}	K_M^{Pyl}, μM
chPylRS	33.24 ± 2.74	21.03 ± 0.15
V31I, T56P, A100E	289.16 ± 11.45	18.42 ± 0.69

The mean values and standard errors were calculated from three replicates.

Supplementary Table 6. Kinetic parameters of the fusions of split chPylRS variants from PACE.

AARS variant	k_{cat}, s^{-1} x 10^{-3}	K_M^{BocK}, mM	K_M^{tRNA}, μM
Fused Split2	20 ± 1	1.68 ± 0.19	3.62 ± 0.51
Fused Split3	33 ± 3	4.90 ± 0.92	3.84 ± 0.34
Fused Split6	19 ± 0.2	1.00 ± 0.05	3.61 ± 0.38

The mean values and standard errors were calculated from three replicates.

Supplementary Table 7. Kinetic parameters of *Mj*TyrRS variants containing mutations from PACE.

AARS variant	**ncAA**	k_{cat}, s^{-1} x 10^{-3}	K_M^{ncAA}, mM	k_{cat}/K_M^{ncAA}, $mM^{-1} \cdot s^{-1}$ x 10^{-3}	Relative catalytic efficiency
p-NFRS	*p*-NF	1.40 ± 0.05	3.68 ± 0.29	0.38	1.00
p-NFRS	*p*-IF	0.87 ± 0.11	2.23 ± 0.46	0.39	1.03
p-NFRS	Phe	0.14 ± 0.003	0.16 ± 0.03	0.875	2.3
Iodo.5	*p*-NF	ND	ND	ND	ND
Iodo.5	*p*-IF	ND	ND	ND	ND
Iodo.1	*p*-IF	1.60 ± 1.27	5.65 ± 1.82	0.28	0.74
Iodo.7	*p*-IF	0.21 ± 0.03	0.92 ± 0.22	0.23	0.61
Iodo.8	*p*-IF	1.00 ± 0.10	3.80 ± 0.84	0.26	0.68

The mean values and standard errors were calculated from three replicates. ND, not determined due to loss of activity upon purification.

Supplementary Table 8. Plasmids used in this work

Plasmid Name	Class (resistance)	Origin	ORF1		ORF2		ORF3		PACE Experiments	Figs
			Prom	[RBS][2] Genes	Prom	Genes	Prom	[RBS] Genes		
pDB007(+)	AP (carb^R)	SC101	P_{T7}	[SD8] gIII, luxAB	P_{ProK}	$tyrT^{Opt}_{CUA}$	–	–	*p*-NFRS	1, 3a, 5c, S3a, S4a
pDB021CH(+)	AP (carb^R)	SC101	P_{T7}	[SD8] gIII, luxAB	P_{ProK}	pylT	–	–	Pyl-1, Pyl-2	1, 4a, S3b
pDB026a	AP (carb^R)	SC101	P_{psp}	[SD8] gIII(P29∗), luxAB	P_{ProK}	$tyrT^{Opt}_{CUA}$	–	–		S3c, S4b
pDB026b	AP (carb^R)	SC101	P_{psp}	[SD8] gIII(P83∗), luxAB	P_{ProK}	$tyrT^{Opt}_{CUA}$	–	–		S3c
pDB026c	AP (carb^R)	SC101	P_{psp}	[SD8] gIII(T177∗), luxAB	P_{ProK}	$tyrT^{Opt}_{CUA}$	–	–		S3c
pDB026d	AP (carb^R)	SC101	P_{psp}	[SD8] gIII(Y184∗), luxAB	P_{ProK}	$tyrT^{Opt}_{CUA}$	–	–		S3c
pDB026e	AP (carb^R)	SC101	P_{psp}	[SD8] gIII(P29∗,Y184∗), luxAB	P_{ProK}	$tyrT^{Opt}_{CUA}$	–	–		S3c
pDB026f	AP (carb^R)	SC101	P_{psp}	[SD8] gIII(P29∗,P83∗,Y184∗), luxAB	P_{ProK}	$tyrT^{Opt}_{CUA}$	–	–		S3c
pDB026g	AP (carb^R)	SC101	P_{psp}	[SD8] gIII(P29∗,P83∗, T177∗,Y184∗), luxAB	P_{ProK}	$tyrT^{Opt}_{CUA}$	–	–		S3c
pJC175e	AP (carb^R)	SC101	P_{psp}	[SD8] gIII, luxAB	–	–	–	–		Activity indep. titers
pDB038	AP (spec^R)	ColE1	P_{psp}	[SD8] gIII(P29∗), luxAB	P_{ProK}	pylT	–	–	Pyl-3	1, 4a, S3d
pDB038a	AP (spec^R)	ColE1	P_{psp}	[SD8] gIII(P29∗,Y184∗), luxAB	P_{ProK}	pylT	–	–	Pyl-3	1, 4a, S3d
pDB038b	AP (spec^R)	ColE1	P_{psp}	[SD8] gIII(P29∗,P83∗,Y184∗), luxAB	P_{ProK}	pylT	–	–	Pyl-3	1, 4a, S3d
pDB007ns2a	AP$^-$ (carb^R)	SC101	P_{psp}	[SD8] gIII	P_{ProK}	$tyrT^{Opt}_{CUA}$	P_{tet}	[SD4] T7RNAP(S12∗,S203∗)	*p*-NFRS	5a-c, S13, S14, S16
pDB036a	AP$^-$ (carb^R)	SC101	P_{psp}	[SD8] gIII	P_{ProK}	$tyrT^{Opt}_{CUA}$	P_{proD}	[SD4] T7RNAP(S12∗,S203∗)	Countersel.	S17
pDB036d	AP$^-$ (carb^R)	SC101	P_{psp}	[SD8] gIII	P_{ProK}	$tyrT^{Opt}_{CUA}$	P_{proA}	[SD4] T7RNAP(S12∗,S203∗)	Countersel.	S17
pDB023f	CP (spec^R)	ColE1	P_{psp}	[SD8] T7RNAP(S12∗,S203∗)	–	–	–	–	Pyl-1, Pyl-2	1, 4a, S3b
pDB023f1	CP (spec^R)	ColE1	P_{psp}	[SD4] T7RNAP(S12∗,S203∗)	–	–	–	–	*p*-NFRS	1, 3a, 5c, S3a, S4a
pDB023k	CP (spec^R)	ColE1	P_{psp}	[SD8] T7RNAP(S12∗,S203∗,S527∗)	–	–	–	–		1, S3b
pDB016	CP$^-$ (spec^R)	ColE1	P_{T7}	[SD8] gIII-neg	–	–	–	–	*p*-NFRS, Countersel.	5a-c, S13, S14, S16, S17
DP4	DP (chlor^R)	cloDF13	P_{psp}	dnaQ926, dam, seqA	P_C	araC	$P_{psp\text{-}tet}$	[sd8] gIII	Pyl-1, Pyl-2, *p*-NFRS	4a, 5c
DP6	DP (chlor^R)	cloDF13	P_{psp}	dnaQ926, dam, seqA, emrR, ugi, cda1	P_C	araC	$P_{psp\text{-}tet}$	[sd8] gIII	Pyl-3	4a
pBAD-sfGFP	EP (carb^R)	pBR322	P_{BAD}	sfGFP-6xHis variant	P_C	araC	–	–		4d-e, S5c-d, S6-S9
pDB005x(-)	EP (carb^R)	SC101	P_{lacZ}	[SD8] chPylRS	P_{ProK}	pylT	P_{T7}	[SD8] luxAB		SF2
pDB007xb(-)	EP (carb^R)	SC101	P_{lacZ}	[SD8] *p*-NFRS	P_{ProK}	$tyrT^{Opt}_{CUA}$	P_{T7}	[SD8] luxAB		SF2
pDB027c	EP (carb^R)	SC101	P_{BAD}	[SD8] luxAB(Y361∗), [SD8] *Mj*TyrRS variant	P_{ProK}	$tyrT^{Opt}_{CUA}$	P_C	araC		3b
pDB032c	EP (carb^R)	SC101	P_{BAD}	[SD8] luxAB(Y361∗), [SD8] PylRS variant	P_{ProK}	pylT	P_C	araC		4b-c, S5a
pDB059c	EP (carb^R)	SC101	P_{BAD}	[SD8] luxAB(Y361∗)	P_C	araC	–	–		S5b,e-f
pDB070	EP (chlor^R)	p15A	P_{tet}	*Mj*TyrRS variant	P_{ProK}	$tyrT^{Opt}_{CUA}$	P_{PN25}	TetR		5d, S15, SS18
pTECH-AcK3RS	EP (chlor^R)	p15A	P_{lpp}	AcK3RS variant	P_{ProK}	pylT	–	–		4e, S7
pTECH-PylRS	EP (chlor^R)	p15A	P_{lpp}	PylRS variant	P_{ProK}	pylT	–	–		4d, S5c-d, S6, S8-S11
pET28b(+)-sfGFP	EP (Kan^R)	pBR322	P_{T7}	sfGFP-6xHis variant	P_I	LacI	–	–		5d, S15, S18
pDB009a	EP (spec^R)	ColE1	P_{tet}	[SD8] wt T7 RNAP						SF2
pDB009b	EP (spec^R)	ColE1	P_{tet}	[SD8] T7 RNAP(S12∗)						SF2
pDB009c	EP (spec^R)	ColE1	P_{tet}	[SD8] T7 RNAP(S203∗)	–	–	–	–		SF2
pDB009d	EP (spec^R)	ColE1	P_{tet}	[SD8] T7 RNAP(S527∗)	–	–	–	–		SF2
pDB009f	EP (spec^R)	ColE1	P_{tet}	[SD8] T7 RNAP(S12∗,S203∗)	–	–	–	–		SF2
pDB009g	EP (spec^R)	ColE1	P_{tet}	[SD8] T7 RNAP(Y250∗)	–	–	–	–		SF2
pDB009h	EP (spec^R)	ColE1	P_{tet}	[SD8] T7 RNAP(Y312∗)	–	–	–	–		SF2
pDB009i	EP (spec^R)	ColE1	P_{tet}	[SD8] T7 RNAP(Y250∗, Y312∗)	–	–	–	–		SF2
pDB009j	EP (spec^R)	ColE1	P_{tet}	[SD8] T7 RNAP(S12∗, S527∗)	–	–	–	–		SF2

pDB060-AcK3RS	EP (spec^R)	ColE1	P_{lpp}	AcK3RS variant	P_{ProK}	pylT	–	–		S5e
pDB060-IFRS	EP (spec^R)	ColE1	P_{lpp}	IFRS variant	P_{ProK}	pylT	–	–		S5f
pDB060-PylRS	EP (spec^R)	ColE1	P_{lpp}	PylRS variant	P_{ProK}	pylT	–	–		S5b
MP4	MP (chlor^R)	cloDF13	P_{psp}	dnaQ926, dam, seqA	P_C	araC	–	–	Pyl-2, *p*-NFRS	1, 3a, 4a, 5c, S16
SP-Kan	SP (kan^R)	M13 f1	P_{gIII}	Kan	–	–	–	–		S4a
SP-chPylRS	SP (none)	M13 f1	P_{gIII}	[SD4] chPyl	–	–	–	–	Pyl-1	1, 4a, S3b,d
SP-MBP-TEV	SP (none)	M13 f1	P_{gIII}	[SD8] MBP-TEV	–	–	–	–		S4b, S13, S14
SP-*p*-NFRS	SP (none)	M13 f1	P_{gIII}	[SD4] *p*-NFRS	–	–	–	–	*p*-NFRS	1, 3a, 5c, S3a,c, S13, S14

The plasmids are categorized by class, antibiotic resistance, origin of replication, and the combination of promoters, ribosome-binding site, and genes. Relevant PACE experiments and figures where these plasmids were used or described are provide.

Supplementary Note 1. DNA sequences of genes used in this study.

a. DNA sequence of **chPylRS**. Codons highlighted in gray (Val-31, Thr-56, His-62, and Ala-100) were mutated to 'ATT', 'CCC', 'TAT', and 'GAG', respectively, in the 'IPYE' variant of the enzyme.

ATGGATAAGAAGCCGCTGGATGTTCTGATCTCTGCGACCGGTCTGTGGATGTCCCGTACCGGCACG
CTGCACAAGATCAAGCACTATGAGGTTTCTCGTTCTAAAATCTACATCGAAATGGCGTGTGGTGACC
ATCTGGTTGTGAACAACTCTCGTTCTTGTCGTACCGCACGTGCATTCCGTCATCATAAATACCGTAAA
ACCTGCAAACGTTGTCGTGTTTCTGACGAAGATATCAACAACTTCCTGACCCGTTCTACCGAAGGCA
AAACCTCTGTTAAAGTTAAAGTTGTTTCTGCGCCGAAAGTGAAAAAAGCGATGCCGAAATCTGTTTCT
CGTGCGCCGAAACCGCTGGAAAATCCGGTTTCTGCGAAAGCGTCTACCGACACCTCTCGTTCTGTTC
CGTCTCCGGCGAAATCTACCCCGAACTCTCCGGTTCCGACCTCTGCAAGTGCCCCCGCACTTACGA
AGAGCCAGACTGACAGGCTTGAAGTCCTGTTAAACCCAAAAGATGAGATTTCCCTGAATTCCGGCAA
GCCTTTCAGGGAGCTTGAGTCCGAATTGCTCTCTCGCAGAAAAAAAGACCTGCAGCAGATCTACGCG
GAAGAAAGGGAGAATTATCTGGGGAAACTCGAGCGTGAAATTACCAGGTTCTTTGTGGACAGGGGTT
TTCTGGAAATAAAATCCCCGATCCTGATCCCTCTTGAGTATATCGAAAGGATGGGCATTGATAATGAT
ACCGAACTTTCAAAACAGATCTTCAGGGTTGACAAGAACTTCTGCCTGAGACCCATGCTTGCTCCAA
ACCTTTACAACTACCTGCGCAAGCTTGACAGGGCCCTGCCTGATCCAATAAAAATTTTTGAAATAGGC
CCATGCTACAGAAAAGAGTCCGACGGCAAAGAACACCTCGAAGAGTTTACCATGCTGAACTTCTGCC
AGATGGGATCGGGATGCACACGGGAAAATCTTGAAAGCATAATTACGGACTTCCTGAACCACCTGG
GAATTGATTTCAAGATCGTAGGCGATTCCTGCATGGTCTATGGGGATACCCTTGATGTAATGCACGG
AGACCTGGAACTTTCCTCTGCAGTAGTCGGACCCATACCGCTTGACCGGGAATGGGGTATTGATAAA
CCCTGGATAGGGGCAGGTTTCGGACTCGAACGCCTTCTAAAGGTTAAACACGACTTTAAAAATATCA
AGAGAGCTGCAAGGTCCGAGTCTTACTATAACGGGATTTCTACCAACCTGTAA

b. DNA sequence of ***Mb*PylRS**. Codons highlighted in gray (Val-31, Thr-56, His-62, and Ala-100) were mutated to 'ATT', 'CCC', 'TAT', and 'GAG', respectively, in the 'IPYE' variant of the enzyme.

ATGGATAAGAAGCCGCTGGATGTTCTGATCTCTGCGACCGGTCTGTGGATGTCCCGTACCGGCACG
CTGCACAAGATCAAGCACTATGAGGTTTCTCGTTCTAAAATCTACATCGAAATGGCGTGTGGTGACC
ATCTGGTTGTGAACAACTCTCGTTCTTGTCGTACCGCACGTGCATTCCGTCATCATAAATACCGTAAA
ACCTGCAAACGTTGTCGTGTTTCTGACGAAGATATCAACAACTTCCTGACCCGTTCTACCGAAGGCA
AAACCTCTGTTAAAGTTAAAGTTGTTTCTGCGCCGAAAGTGAAAAAAGCGATGCCGAAATCTGTTTCT
CGTGCGCCGAAACCGCTGGAAAATCCGGTTTCTGCGAAAGCGTCTACCGACACCTCTCGTTCTGTTC
CGTCTCCGGCGAAATCTACCCCGAACTCTCCGGTTCCGACCTCTGCGCCGGCGCCGTCTCTGACCC
GTTCTCAGCTGGATCGTGTTGAAGCGCTGCTGTCTCCGGAAGATAAAATCTCTCTGAACATCGCGAA
ACCGTTCCGTGAACTGGAATCTGAACTGGTTACCCGTCGTAAAAACGATTTCCAGCGTCTGTACACC
AACGATCGTGAAGACTACCTGGGTAAACTGGAACGTGACATCACCAAATTCTTCGTTGACCGTGATT
TCCTGGAAATCAAATCTCCGATCCTGATCCCGGCGGAATACGTTGAACGTATGGGTATCAACAACGA
TACCGAACTGTCTAAACAGATCTTCCGTGTTGATAAAAACCTGTGCCTGCGTCCGATGCTGGCGCCG
ACCCTGTACAACTATCTGCGTAAACTGGATCGTATCCTGCCGGACCCGATCAAAATCTTCGAAGTTG
GTCCGTGCTACCGTAAAGAATCTGACGGTAAAGAACACCTGGAAGAGTTCACCATGGTGAACTTCTG
CCAGATGGGTTCTGGTTGCACCCGTGAGAACCTGGAATCTCTGATCAAAGAATTTCTGGACTACCTG
GAAATCGACTTCGAAATCGTTGGTGACTCCTGCATGGTGTACGGTGATACCCTGGACATCATGCACG
GTGACCTGGAACTGTCTTCTGCGGTTGTTGGTCCGGTTCCGCTGGATCGTGAATGGGGTATCGACA
AACCGTGGATCGGTGCGGGTTTCGGTCTGGAACGTCTGCTGAAAGTTATGCACGGTTTCAAAAACAT
CAAACGTGCGTCTCGTTCTGAATCTTACTACAACGGTATCTCTACCAACCTGTAA

c. DNA sequence of ***Mm*PylRS**. Codons highlighted in gray (Val-31, Thr-56, His-62, and Ala-100) were mutated to 'ATT', 'CCC', 'TAT', and 'GAG', respectively, in the 'IPYE' variant of the enzyme.

ATGGATAAAAAACCACTAAACACTCTGATATCTGCAACCGGGCTCTGGATGTCCAGGACCGGAACAA
TTCATAAAATAAAACACCACGAAGTCTCTCGAAGCAAAATCTATATTGAAATGGCATGCGGAGACCAC
CTTGTTGTAAACAACTCCAGGAGCAGCAGGACTGCAAGAGCGCTCAGGCACCACAAATACAGGAAG
ACCTGCAAACGCTGCAGGGTTTCGGATGAGGATCTCAATAAGTTCCTCACAAAGGCAAACGAAGACC
AGACAAGCGTAAAAGTCAAGGTCGTTTCTGCCCCTACCAGAACGAAAAAGGCAATGCCAAAATCCGT
TGCGAGAGCCCCGAAACCTCTTGAGAATACAGAAGCGGCACAGGCTCAACCTTCTGGATCTAAATTT
TCACCTGCGATACCGGTTTCCACCCAAGAGTCAGTTTCTGTCCCGGCATCTGTTTCAACATCAATATC
AAGCATTTCTACAGGAGCAACTGCATCCGCACTGGTAAAAGGGAATACGAATCCCATTACATCCATG
TCTGCCCCTGTTCAGGCAAGTGCCCCCGCACTTACGAAGAGCCAGACTGACAGGCTTGAAGTCCTG
TTAAACCCAAAAGATGAGATTTCCCTGAATTCCGGCAAGCCTTTCAGGGAGCTTGAGTCCGAATTGC
TCTCTCGCAGAAAAAAAGACCTGCAGCAGATCTACGCGGAAGAAAGGGAGAATTATCTGGGGAAACT
CGAGCGTGAAATTACCAGGTTCTTTGTGGACAGGGGTTTTCTGGAAATAAAATCCCCGATCCTGATC
CCTCTTGAGTATATCGAAAGGATGGGCATTGATAATGATACCGAACTTTCAAAACAGATCTTCAGGGT
TGACAAGAACTTCTGCCTGAGACCCATGCTTGCTCCAAACCTTTACAACTACCTGCGCAAGCTTGAC
AGGGCCCTGCCTGATCCAATAAAAATTTTTGAAATAGGCCCATGCTACAGAAAAGAGTCCGACGGCA
AAGAACACCTCGAAGAGTTTACCATGCTGAACTTCTGCCAGATGGGATCGGGATGCACACGGGAAA
ATCTTGAAAGCATAATTACGGACTTCCTGAACCACCTGGGAATTGATTTCAAGATCGTAGGCGATTCC
TGCATGGTCTATGGGGATACCCTTGATGTAATGCACGGAGACCTGGAACTTTCCTCTGCAGTAGTCG
GACCCATACCGCTTGACCGGGAATGGGGTATTGATAAACCCTGGATAGGGGCAGGTTTCGGGCTCG
AACGCCTTCTAAAGGTTAAACACGACTTTAAAAATATCAAGAGAGCTGCAAGGTCCGAGTCTTACTAT
AACGGGATTTCTACCAACCTGTAA

d. DNA sequence of **chAcK3RS**. Codons highlighted in gray (Val-31, Thr-56, His-62, and Ala-100) were mutated to 'ATT', 'CCC', 'TAT', and 'GAG', respectively, in the 'IPYE' variant of the enzyme.

ATGGATAAGAAGCCGCTGGATGTTCTGATCTCTGCGACCGGTCTGTGGATGTCCCGTACCGGCACG
CTGCACAAGATCAAGCACTATGAGGTTTCTCGTTCTAAAATCTACATCGAAATGGCGTGTGGTGACC
ATCTGGTTGTGAACAACTCTCGTTCTTGTCGTACCGCACGTGCATTCCGTCATCATAAATACCGTAAA
ACCTGCAAACGTTGTCGTGTTTCTGGTGAAGATATCAACAACTTCCTGACCCGTTCTACCGAAGGCA
AAACCTCTGTTAAAGTTAAAGTTGTTTCTGCGCCGAAAGTGAAAAAAGCGATGCCGAAATCTGTTTCT
CGTGCGCCGAAACCGCTGGAAAATCCGGTTTCTGCGAAAGCGTCTACCGACACCTCTCGTTCTGTTC
CGTCTCCGGCGAAATCTACCCCGAACTCTCCGGTTCCGACCTCTGCAAGTGCCCCCGCACTTACGA
AGAGCCAGACTGACAGGCTTGAAGTCCTGTTAAACCCAAAAGATGAGATTTCCCTGAATTCCGGCAA
GCCTTTCAGGGAGCTTGAGTCCGAATTGCTCTCTCGCAGAAAAAAAGACCTGCAGCAGATCTACGCG
GAAGAAAGGGAGAATTATCTGGGGAAACTCGAGCGTGAAATTACCAGGTTCTTTGTGGACAGGGGTT
TTCTGGAAATAAAATCCCCGATCCTGATCCCTCTTGAGTATATCGAAAGGATGGGCATTGATAATGAT
ACCGAACTTTCAAAACAGATCTTCAGGGTTGACAAGAACTTCTGCCTGAGACCCATGATGGCTCCAA
ACATTTTTAACTACGCTCGCAAGCTTGACAGGGCCCTGCCTGATCCAATAAAAATTTTTGAAATAGGC
CCATGCTACAGAAAAGAGTCCGACGGCAAAGAACACCTCGAAGAGTTTACCATGCTGAACTTCTTTC
AGATGGGATCGGGATGCACACGGGAAAATCTTGAAAGCATAATTACGGACTTCCTGAACCACCTGG
GAATTGATTTCAAGATCGTAGGCGATTCCTGCATGGTCTATGGGGATACCCTTGATGTAATGCACGG
AGACCTGGAACTTTCCTCTGCAGTAGTCGGACCCATACCGCTTGACCGGGAATGGGGTATTGATAAA
CCCTGGATAGGGGCAGGTTTCGGACTCGAACGCCTTCTAAAGGTTAAACACGACTTTAAAAATATCA
AGAGAGCTGCAAGGTCCGAGTCTTACTATAACGGGATTTCTACCAACCTGTAA

e. DNA sequence of ***Mb*AcK3RS**. Codons highlighted in gray (Val-31, Thr-56, His-62, and Ala-100) were mutated to 'ATT', 'CCC', 'TAT', and 'GAG', respectively, in the 'IPYE' variant of the enzyme.

ATGGATAAGAAGCCGCTGGATGTTCTGATCTCTGCGACCGGTCTGTGGATGTCCCGTACCGGCACG
CTGCACAAGATCAAGCACTATGAGGTTTCTCGTTCTAAAATCTACATCGAAATGGCGTGTGGTGACC
ATCTGGTTGTGAACAACTCTCGTTCTTGTCGTACCGCACGTGCATTCCGTCATCATAAATACCGTAAA
ACCTGCAAACGTTGTCGTGTTTCTGGTGAAGATATCAACAACTTCCTGACCCGTTCTACCGAAGGCA
AAACCTCTGTTAAAGTTAAAGTTGTTTCTGCGCCGAAAGTGAAAAAAGCGATGCCGAAATCTGTTTCT
CGTGCGCCGAAACCGCTGGAAAATCCGGTTTCTGCGAAAGCGTCTACCGACACCTCTCGTTCTGTTC
CGTCTCCGGCGAAATCTACCCCGAACTCTCCGGTTCCGACCTCTGCGCCGGCGCCGTCTCTGACCC
GTTCTCAGCTGGATCGTGTTGAAGCGCTGCTGTCTCCGGAAGATAAAATCTCTCTGAACATCGCGAA
ACCGTTCCGTGAACTGGAATCTGAACTGGTTACCCGTCGTAAAAACGATTTCCAGCGTCTGTACACC
AACGATCGTGAAGACTACCTGGGTAAACTGGAACGTGACATCACCAAATTCTTCGTTGACCGTGATT
TCCTGGAAATCAAATCTCCGATCCTGATCCCGGCGGAATACGTTGAACGTATGGGTATCAACAACGA
TACCGAACTGTCTAAACAGATCTTCCGTGTTGATAAAAACCTGTGCCTGCGTCCGATGATGGCGCCG
ACCATTTTTAACTATGCTCGTAAACTGGATCGTATCCTGCCGGACCCGATCAAAATCTTCGAAGTTGG
TCCGTGCTACCGTAAAGAATCTGACGGTAAAGAACACCTGGAAGAGTTCACCATGGTGAACTTCTTT
CAGATGGGTTCTGGTTGCACCCGTGAGAACCTGGAATCTCTGATCAAAGAATTTCTGGACTACCTGG
AAATCGACTTCGAAATCGTTGGTGACTCCTGCATGGTGTACGGTGATACCCTGGACATCATGCACGG
TGACCTGGAACTGTCTTCTGCGGTTGTTGGTCCGGTTCCGCTGGATCGTGAATGGGGTATCGACAAA
CCGTGGATCGGTGCGGGTTTCGGTCTGGAACGTCTGCTGAAAGTTATGCACGGTTTCAAAAACATCA
AACGTGCGTCTCGTTCTGAATCTTACTACAACGGTATCTCTACCAACCTGTAA

f. DNA sequence of ***Mm*AcK3RS**. Codons highlighted in gray (Val-31, Thr-56, His-62, and Ala-100) were mutated to 'ATT', 'CCC', 'TAT', and 'GAG', respectively, in the 'IPYE' variant of the enzyme.

ATGGATAAAAAACCACTAAACACTCTGATATCTGCAACCGGGCTCTGGATGTCCAGGACCGGAACAA
TTCATAAAATAAAACACCACGAAGTCTCTCGAAGCAAAATCTATATTGAAATGGCATGCGGAGACCAC
CTTGTTGTAAACAACTCCAGGAGCAGCAGGACTGCAAGAGCGCTCAGGCACCACAAATACAGGAAG
ACCTGCAAACGCTGCAGGGTTTCGGGTGAGGATCTCAATAAGTTCCTCACAAAGGCAAACGAAGAC
CAGACAAGCGTAAAAGTCAAGGTCGTTTCTGCCCCTACCAGAACGAAAAAGGCAATGCCAAAATCCG
TTGCGAGAGCCCCGAAACCTCTTGAGAATACAGAAGCGGCACAGGCTCAACCTTCTGGATCTAAATT
TTCACCTGCGATACCGGTTTCCACCCAAGAGTCAGTTTCTGTCCCGGCATCTGTTTCAACATCAATAT
CAAGCATTTCTACAGGAGCAACTGCATCCGCACTGGTAAAAGGGAATACGAATCCCATTACATCCAT
GTCTGCCCCTGTTCAGGCAAGTGCCCCCGCACTTACGAAGAGCCAGACTGACAGGCTTGAAGTCCT
GTTAAACCCAAAAGATGAGATTTCCCTGAATTCCGGCAAGCCTTTCAGGGAGCTTGAGTCCGAATTG
CTCTCTCGCAGAAAAAAAGACCTGCAGCAGATCTACGCGGAAGAAAGGGAGAATTATCTGGGGAAA
CTCGAGCGTGAAATTACCAGGTTCTTTGTGGACAGGGGTTTTCTGGAAATAAAATCCCCGATCCTGA
TCCCTCTTGAGTATATCGAAAGGATGGGCATTGATAATGATACCGAACTTTCAAAACAGATCTTCAGG
GTTGACAAGAACTTCTGCCTGAGACCCATGATGGCTCCAAACATTTTTAACTACGCTCGCAAGCTTGA
CAGGGCCCTGCCTGATCCAATAAAAATTTTTGAAATAGGCCCATGCTACAGAAAAGAGTCCGACGGC
AAAGAACACCTCGAAGAGTTTACCATGCTGAACTTCTTTCAGATGGGATCGGGATGCACACGGGAAA
ATCTTGAAAGCATAATTACGGACTTCCTGAACCACCTGGGAATTGATTTCAAGATCGTAGGCGATTCC
TGCATGGTCTATGGGGATACCCTTGATGTAATGCACGGAGACCTGGAACTTTCCTCTGCAGTAGTCG
GACCCATACCGCTTGACCGGGAATGGGGTATTGATAAACCCTGGATAGGGGCAGGTTTCGGGCTCG
AACGCCTTCTAAAGGTTAAACACGACTTTAAAAATATCAAGAGAGCTGCAAGGTCCGAGTCTTACTAT
AACGGGATTTCTACCAACCTGTAA

g. DNA sequence of **chIFRS**. Codons highlighted in gray (Val-31, Thr-56, His-62, and Ala-100) were mutated to 'ATT', 'CCC', 'TAT', and 'GAG', respectively, in the 'IPYE' variant of the enzyme.

ATGGATAAGAAGCCGCTGGATGTTCTGATCTCTGCGACCGGTCTGTGGATGTCCCGTACCGGCACG
CTGCACAAGATCAAGCACTATGAGGTTTCTCGTTCTAAAATCTACATCGAAATGGCGTGTGGTGACC
ATCTGGTTGTGAACAACTCTCGTTCTTGTCGTACCGCACGTGCATTCCGTCATCATAAATACCGTAAA
ACCTGCAAACGTTGTCGTGTTTCTGACGAAGATATCAACAACTTCCTGACCCGTTCTACCGAAGGCA
AAACCTCTGTTAAAGTTAAAGTTGTTTCTGCGCCGAAAGTGAAAAAAGCGATGCCGAAATCTGTTTCT
CGTGCGCCGAAACCGCTGGAAAATCCGGTTTCTGCGAAAGCGTCTACCGACACCTCTCGTTCTGTTC
CGTCTCCGGCGAAATCTACCCCGAACTCTCCGGTTCCGACCTCTGCAAGTGCCCCCGCACTTACGA
AGAGCCAGACTGACAGGCTTGAAGTCCTGTTAAACCCAAAAGATGAGATTTCCCTGAATTCCGGCAA
GCCTTTCAGGGAGCTTGAGTCCGAATTGCTCTCTCGCAGAAAAAAAGACCTGCAGCAGATCTACGCG
GAAGAAAGGGAGAATTATCTGGGGAAACTCGAGCGTGAAATTACCAGGTTCTTTGTGGACAGGGGTT
TTCTGGAAATAAAATCCCCGATCCTGATCCCTCTTGAGTATATCGAAAGGATGGGCATTGATAATGAT
ACCGAACTTTCAAAACAGATCTTCAGGGTTGACAAGAACTTCTGCCTGAGACCCATGCTTGCTCCAA
ACCTTTACAACTACCTGCGCAAGCTTGACAGGGCCCTGCCTGATCCAATAAAAATTTTTGAAATAGGC
CCATGCTACAGAAAAGAGTCCGACGGCAAAGAACACCTCGAAGAGTTTACCATGCTGTCGTTCATTC
AGATGGGATCGGGATGTACACGGGAAAATCTTGAAAGCATAATTACGGACTTCCTGAACCACCTGGG
AATTGATTTCAAGATCGTAGGCGATTCCTGCATGGTCTATGGGGATACCCTTGATGTAATGCACGGA
GACCTGGAACTTTCCTCTGCAGTAGTCGGACCCATACCGCTTGACCGGGAATGGGGTATTGATAAAC
CCTGGATAGGGGCAGGTTTCGGGCTCGAACGCCTTCTAAAGGTTAAACACGACTTTAAAAATATCAA
GAGAGCTGCAAGGTCCGAGTCTTACTATAACGGGATTTCTACCAACCTGTAA

h. DNA sequence of PACE-evolved chPylRS variant, **Split1**. The in-frame, premature stop codon of the split enzyme is highlighted in yellow, and the position of translational reinitiation, corresponding to Met-107 of chPylRS, is highlighted in blue. In the **Spit1'** variant, codons highlighted in gray were reverted back to 'GTT', 'ACC', 'CAT', and 'GCG', respectively.

ATGGATAAGAAGCCGCTGGATGTTCTGATCTCTGCGACCGGTCTGTGGATGTCCCGTACCGGCACG
CTGCACAAGATCAAGCACTATGAGATTTCTCGTTCTAAAATCTACATCGAAATGGCGTGTGGTGACCA
TCTGGTTGTGAACAACTCTCGTTCTTGTCGTCCCGCACGTGCATTCCGTTATCATAAATACCGTAAAA
CCTGCAAACGTTGTCGTGTTTCTGACGAAGATATCAACAACTTCCTGACCCGTTCTACCGAAGGCAA
AACCTCTGTTAAAGTTAAAGCTGTTCTGAGCCGAAAGTGAAAAAAGCGATGCCGAAATCTGTTTCTCG
TGCGCCGAAACCGCTGGAAAATCCGGTTTCTGCGAAAGCGTCTACCGACACCTCTCGTTCTGTTCCG
TCTCCGGCGAAATCTACCCCGAACTCTCCGGTTCCGACCTCTGCAAGTGCCCCCGCACTTACGAAG
AGCCAGACTGACAGGCTTGAAGTCCTGTTAAACCCAAAAGATGAGATTTCCCTGAATTCCGGCAAGC
CTTTCAGGGAGCTTGAGTCCGAATTGCTCTCTCGCAGAAAAAAAGACCTGCAGCAGATCTACGCGGA
AGAAAGGGAGAATTATCTGGGGAAACTCGAGCGTGAAATTACCAGGTTCTTTGTGGACAGGGGTTTT
CTGGAAATAAAATCCCCGATCCTGATCCCTCTTGAGTATATCGAAAGGATGGGCATTGATAATGATAC
CGAACTTTCAAAACAGATCTTCAGGGTTGACAAGAACTTCTGCCTGAGACCCATGCTTGCTCCAAAC
CTTTACAACTACCTGCGCAAGCTTGACAGGGCCCTGCCTGATCCAATAAAAATTTTTGAAATAGGCCC
ATGCTACAGAAAAGAGTCCGACGGCAAAGAACACCTCGAAGAGTTTACCATGCTGAACTTCTGCCAG
ATGGGATCGGGATGCACACGGGAAAATCTTGAAAGCATAATTACGGACTTCCTGAACCACCTGGGAA
TTGATTTCAAGATCGTAGGCGATTCCTGCATGGTCTATGGGGATACCCTTGATGTAATGCACGGAGA
CCTGGAACTTTCCTCTGCAGTAGTCGGACCCATACCGCTTGACCGGGAATGGGGTATTGATAAACCC
TGGATAGGGGCAGGTTTCGGGCTCGAACGCCTTCTAAAGGTTAAACACGACTTTAAAAATATCAAGA
GAGCTGCAAGGTCCGAGTCTTACTATAACGGGATTTCTACCAACCTGTAA

i. DNA sequence of PACE-evolved chPylRS variant, **Split2**. The in-frame, premature stop codon of the split enzyme is highlighted in yellow, and the position of translational reinitiation, corresponding to Met-107 of chPylRS, is highlighted in blue. In the **Spit2'** variant, codons highlighted in gray were reverted back to 'GTT', 'ACC', 'CAT', and 'GCG', respectively.

ATGGATAAGAAGCCGCTGGATGTTCTGATCTCTGCGACCGGTCTGTGGATGTCCCGTACCGGCACG
CTGCACAAGATCAAGCACTATGAGATTTCTCGTTCTAAAATCTACATCGAAATGGCGTGTGGTGACCA
TCTGGTTGTGAACAACTCTCGTTCTTGTCGTCCCGCACGTGCATTCCGTTATCATAAATACCGTAAAA
CCTGCAAACGTTGTCGTGTTTCTGACGAAGATATCAACAACTTCCTGACCCGTTCTACCGAAGGCAA
AACCTCTGTTAAAGTTAAAGTTGTTCTGAGCCGAAAGTGAAAAAAGCGATGCCGAAATCTGTTTCTCG
TGCGCCGAAACCGCTGGAAAATCCGGTTTCTGCGAAAGCGTCTACCGACACCTCTCGTTCTGTTCCG
TCTCCGGCGAAATCTACCCCGAACTCTCCGGTTCCGACCTCTGCAAGTGCCCCCGCACTTACGAAG
AGCCAGACTGACAGGCTTGAAGTCCTGTTAAACCCAAAAGATGAGATTTCCCTGAATTCCGGCAAGC
CTTTCAGGGAGCTTGAGTCCGAATTGCTCTCTCGCAGAAAAAAAGACCTGCAGCAGATCTACGCGGA
AGAAAGGGAGAATTATCTGGGGAAACTCGAGCGTGAAATTACCAGGTTCTTTGTGGACAGGGGTTTT
CTGGAAATAAAATCCCCGATCCTGATCCCTCTTGAGTATATCGAAAGGATGGGCATTGATAATGATAC
CGAACTTTCAAAACAGATCTTCAGGGTTGACAAGAACTTCTGCCTGAGACCCATGCTTGCTCCAAAC
CTTTACAACTACCTGCGCAAGCTTGACAGGGCCCTGCCTGATCCAATAAAAATTTTTGAAATAGGCCC
ATGCTACAGAAAAGAGTCCGACGGCAAAGAACACCTCGAAGAGTTTACCATGCTGAACTTCTGCCAG
ATGGGATCGGGATGCACACGGGAAAATCTTGAAAGCATAATTACGGACTTCCTGAACCACCTGGGAA
TTGATTTCAAGATCGTAGGCGATTCCTGCATGGTCTATGGGGATACCCTTGATGTAATGCACGGAGA
CCTGGAACTTTCCTCTGCAGTAGTCGGACCCATACCGCTTGACCGGGAATGGGGTATTGATAAACCC
TGGATAGGGGCAGGTTTCGGGCTCGAACGCCTTCTAAAGGTTAAACACGACTTTAAAAATATCAAGA
GAGCTGCAAGGTCCGAGTCTTACTATAACGGGATTTCTACCAACCTGTAA

j. DNA sequence of PACE-evolved chPylRS variant, **Split3**. The in-frame, premature stop codon of the split enzyme is highlighted in yellow, and the position of translational reinitiation, corresponding to Met-107 of chPylRS, is highlighted in blue. In the **Spit3'** variant, codons highlighted in gray or underlined were reverted back to 'GTT', 'ACC', 'CAT', and 'GCG', respectively.

ATGGATAAGAAGCCGCTGGATGTTCTGATCTCTGCGACCGGTCTGTGGATGTCCCGTACCGGCACG
CTGCACAAGATCAAGCACTATGAGATTTCTCGTTCTAAAATCTACATCGAAATGGCGTGTGGTGACCA
TCTGGTTGTGAACAACTCTCGTTCTTGTCGTCCCGCACGTGCATTCCGTTATCATAAATACCGTAAAA
CCTGCAAACGTTGTCGTGTTTCTGACGAAGATATCAACAACTTCCTGACCCGTTCTACCGAAGGCAA
AACCTCTGTTAAAGTTAAAGTTGTTTTCTGAGCCGAAAGTGAAAAAAGCGATGCCGAAATCTGTTTCT
CGTGCGCCGAAACCGCTGGAAAATCCGGTTTCTGCGAAAGCGTCTACCGACACCTCTCGTTCTGTTC
CGTCTCCGGCGAAATCTACCCCGAACTCTCCGGTTCCGACCTCTGCAAGTGCCCCCGCACTTACGA
AGAGCCAGACTGACAGGCTTGAAGTCCTGTTAAACCCAAAAGATGAGATTTCCCTGAATTCCGGCAA
GCCTTTCAGGGAGCTTGAGTCCGAATTGCTCTCTCGCAGAAAAAAAGACCTGCAGCAGATCTACGCG
GAAGAAAGGGAGAATTATCTGGGGAAACTCGAGCGTGAAATTACCAGGTTCTTTGTGGACAGGGGTT
TTCTGGAAATAAAATCCCCGATCCTGATCCCTCTTGAGTATATCGAAAGGATGGGCATTGATAATGAT
ACCGAACTTTCAAAACAGATCTTCAGGGTTGACAAGAACTTCTGCCTGAGACCCATGCTTGCTCCAA
ACCTTTACAACTACCTGCGCAAGCTTGACAGGGCCCTGCCTGATCCAATAAAAATTTTTGAAATAGGC
CCATGCTACAGAAAAGAGTCCGACGGCAAAGAACACCTCGAAGAGTTTACCATGCTGAACTTCTGCC
AGATGGGATCGGGATGCACACGGGAAAATCTTGAAAGCATAATTACGGACTTCCTGAACCACCTGG
GAATTGATTTCAAGATCGTAGGCGATTCCTGCATGGTCTATGGGGATACCCTTGATGTAATGCACGG
AGACCTGGAACTTTCCTCTGCAGTAGTCGGACCCATACCGCTTGACCGGGAATGGGGTATTGATAAA
CCCTGGATAGGGGCAGGTTTCGGGCTCGAACGCCTTCTAAAGGTTAAACACGACTTTAAAAATATCA
AGAGAGCTGCAAGGTCCGAGTCTTACTATAACGGGATTTCTACCAACCTGTAA

k. DNA sequence of PACE-evolved chPylRS variant, **Split4**. The in-frame, premature stop codon of the split enzyme is highlighted in yellow, and the position of translational reinitiation, corresponding to Met-107 of chPylRS, is highlighted in blue. In the **Spit4'** variant, codons highlighted in gray were reverted back to 'GTT', 'ACC', 'CAT', and 'GCG', respectively.

ATGGATAAGAAGCCGCTGGATGTTCTGATCTCTGCGACCGGTCTGTGGATGTCCCGTACCGGCACG
CTGCACAAGATCAAGCACTATGAGATTTCTCGTTCTAAAATCTACATCGAAATGGCGTGTGGTGACCA
TCTGGTTGTGAACAACTCTCGTTCTTGTCGTCCCGCACGTGCATTCCGTTATCATAAATACCGTAAAA
CCTGCAAACGTTGTCGTGTTTCTGACGAAGATATCAACAACTTCCTGACCCGTTCTACCGAAGGCTA
AACCTCTGTTAAAGTTAAAGTTGTTTCTGAGCCGAAAGTGAAAAAAGCGATGCCGAAATCTGTTTCTC
GTGCGCCGAAACCGCTGGAAAATCCGGTTTCTGCGAAAGCGTCTACCGACACCTCTCGTTCTGTTCC
GTCTCCGGCGAAATCTACCCCGAACTCTCCGGTTCCGACCTCTGCAAGTGCCCCCGCACTTACGAA
GAGCCAGACTGACAGGCTTGAAGTCCTGTTAAACCCAAAAGATGAGATTTCCCTGAATTCCGGCAAG
CCTTTCAGGGAGCTTGAGTCCGAATTGCTCTCTCGCAGAAAAAAAGACCTGCAGCAGATCTACGCGG
AAGAAAGGGAGAATTATCTGGGGAAACTCGAGCGTGAAATTACCAGGTTCTTTGTGGACAGGGGTTT
TCTGGAAATAAAATCCCCGATCCTGATCCCTCTTGAGTATATCGAAAGGATGGGCATTGATAATGATA
CCGAACTTTCAAAACAGATCTTCAGGGTTGACAAGAACTTCTGCCTGAGACCCATGCTTGCTCCAAA
CCTTTACAACTACCTGCGCAAGCTTGACAGGGCCCTGCCTGATCCAATAAAAATTTTTGAAATAGGCC
CATGCTACAGAAAAGAGTCCGACGGCAAAGAACACCTCGAAGAGTTTACCATGCTGAACTTCTGCCA
GATGGGATCGGGATGCACACGGGAAAATCTTGAAAGCATAATTACGGACTTCCTGAACCACCTGGG
AATTGATTTCAAGATCGTAGGCGATTCCTGCATGGTCTATGGGGATACCCTTGATGTAATGCACGGA
GACCTGGAACTTTCCTCTGCAGTAGTCGGACCCATACCGCTTGACCGGGAATGGGGTATTGATAAAC
CCTGGATAGGGGCAGGTTTCGGGCTCGAACGCCTTCTAAAGGTTAAACACGACTTTAAAAATATCAA
GAGAGCTGCAAGGTCCGAGTCTTACTATAACGGGATTTCTACCAACCTGTAA

l. DNA sequence of PACE-evolved chPylRS variant, **Split5**. The in-frame, premature stop codon of the split enzyme is highlighted in yellow, and the position of translational reinitiation, corresponding to Met-107 of chPylRS, is highlighted in blue. In the **Spit5'** variant, codons highlighted in gray were reverted back to 'GTT', 'ACC', 'CAT', and 'GCG', respectively.

ATGGATAAGAAGCCGCTGGATGTTCTGATCTCTGCGACCGGTCTGTGGATGTCCCGTACCGGCACG
CTGCACAAGATCAAGCACTATGAGATTTCTCGTTCTAAAATCTACATCGAAATGGCGTGTGGTGACCA
TCTGGTTGTGAACAACTCTCGTTCTTGTCGTCCCGCACGTGCATTCCGTTATCATAAATACCGTAAAA
CCTGCAAACGTTGTCGTGTTTCTGACGAAGATATCAACAACTTCCTGACCCGTTCTACCGAAGGCAA
AACCTCTGTTAAAGTTAAAGTTGTTTCTGAGCGAAAGTGAAAAAAGCGATGCCGAAATCTGTTTCTCG
TGCGCCGAAACCGCTGGAAAATCCGGTTTCTGCGAAAGCGTCTACCGACACCTCTCGTTCTGTTCCG
TCTCCGGCGAAATCTACCCCGAACTCTCCGGTTCCGACCTCTGCAAGTGCCCCCGCACTTACGAAG
AGCCAGACTGACAGGCTTGAAGTCCTGTTAAACCCAAAAGATGAGATTTCCCTGAATTCCGGCAAGC
CTTTCAGGGAGCTTGAGTCCGAATTGCTCTCTCGCAGAAAAAAAGACCTGCAGCAGATCTACGCGGA
AGAAAGGGAGAATTATCTGGGGAAACTCGAGCGTGAAATTACCAGGTTCTTTGTGGACAGGGGTTTT
CTGGAAATAAAATCCCCGATCCTGATCCCTCTTGAGTATATCGAAAGGATGGGCATTGATAATGATAC
CGAACTTTCAAAACAGATCTTCAGGGTTGACAAGAACTTCTGCCTGAGACCCATGCTTGCTCCAAAC
CTTTACAACTACCTGCGCAAGCTTGACAGGGCCCTGCCTGATCCAATAAAAATTTTTGAAATAGGCCC
ATGCTACAGAAAAGAGTCCGACGGCAAAGAACACCTCGAAGAGTTTACCATGCTGAACTTCTGCCAG
ATGGGATCGGGATGCACACGGGAAAATCTTGAAAGCATAATTACGGACTTCCTGAACCACCTGGGAA
TTGATTTCAAGATCGTAGGCGATTCCTGCATGGTCTATGGGGATACCCTTGATGTAATGCACGGAGA
CCTGGAACTTTCCTCTGCAGTAGTCGGACCCATACCGCTTGACCGGGAATGGGGTATTGATAAACCC
TGGATAGGGGCAGGTTTCGGGCTCGAACGCCTTCTAAAGGTTAAACACGACTTTAAAAATATCAAGA
GAGCTGCAAGGTCCGAGTCTTACTATAACGGGATTTCTACCAACCTGTAA

m. DNA sequence of PACE-evolved chPylRS variant, **Split6**. This split enzyme contained several in-frame, premature stop codons (highlighted in yellow) between the frameshift and the position of translational reinitiation, corresponding to Met-107 of chPylRS highlighted in blue. In the **Spit6'** variant, codons highlighted in gray or underlined were reverted back to 'GTT', 'ACC', 'CAT', and 'GCG', respectively.

ATGGATAAGAAGCCGCTGGATGTTCTGATCTCTGCGACCGGTCTGTGGATGTCCCGTACCGGCACG
CTGCACAAGATCAAGCACTATGAGATTTCTCGTTCTAAAATCTACATCGAAATGGCGTGTGGTGACCA
TCTGGTTGTGAACAACTCTCGTTCTTGTCGTCCCGCACGTGCATTCCGTTATCATAAATACCGTAAAA
CCTGCAAACGTTGTCGTGTTTCTGACGAAGATATCAACAACTTCCTGACCCGTTCTACCGAAGGCAA
AACCCTCTGTTAAAGTTAAAGTTGTTTCTGAGCCGAAAGTGAAAAAAGCGATGCCGAAATCTGTTTCT
CGTGCGCCGAAACCGCTGGAAAATCCGGTTTCTGCGAAAGCGTCTACCGACACCTCTCGTTCTGTTC
CGTCTCCGGCGAAATCTACCCCGAACTCTCCGGTTCCGACCTCTGCAAGTGCCCCCGCACTTACGA
AGAGCCAGACTGACAGGCTTGAAGTCCTGTTAAACCCAAAAGATGAGATTTCCCTGAATTCCGGCAA
GCCTTTCAGGGAGCTTGAGTCCGAATTGCTCTCTCGCAGAAAAAAAGACCTGCAGCAGATCTACGCG
GAAGAAAGGGAGAATTATCTGGGGAAACTCGAGCGTGAAATTACCAGGTTCTTTGTGGACAGGGGTT
TTCTGGAAATAAAATCCCCGATCCTGATCCCTCTTGAGTATATCGAAAGGATGGGCATTGATAATGAT
ACCGAACTTTCAAAACAGATCTTCAGGGTTGACAAGAACTTCTGCCTGAGACCCATGCTTGCTCCAA
ACCTTTACAACTACCTGCGCAAGCTTGACAGGGCCCTGCCTGATCCAATAAAAATTTTTGAAATAGGC
CCATGCTACAGAAAAGAGTCCGACGGCAAAGAACACCTCGAAGAGTTTACCATGCTGAACTTCTGCC
AGATGGGATCGGGATGCACACGGGAAAATCTTGAAAGCATAATTACGGACTTCCTGAACCACCTGG
GAATTGATTTCAAGATCGTAGGCGATTCCTGCATGGTCTATGGGGATACCCTTGATGTAATGCACGG
AGACCTGGAACTTTCCTCTGCAGTAGTCGGACCCATACCGCTTGACCGGGAATGGGGTATTGATAAA
CCCTGGATAGGGGCAGGTTTCGGGCTCGAACGCCTTCTAAAGGTTAAACACGACTTTAAAAATATCA
AGAGAGCTGCAAGGTCCGAGTCTTACTATAACGGGATTTCTACCAACCTGTAA

n. DNA sequence of ***p*-NFRS**.

ATGGACGAATTTGAAATGATAAAGAGAAACACATCTGAAATTATCAGCGAGGAAGAGTTAAGAGAGG
TTTTAAAAAAAAGATGAAAAATCTGCTCTGATAGGTTTTGAACCAAGTGGTAAAATACATTTAGGGCATT
ATCTCCAAATAAAAAAGATGATTGATTTACAAAATGCTGGATTTGATATAATTATATTGTTGGCTGATTT
ACACGCCTATTTAAACCAGAAAGGAGAGTTGGATGAGATTAGAAAAATAGGAGATTATAACAAAAAAG
TTTTTGAAGCAATGGGGTTAAAGGCAAAATATGTTTATGGAAGTTCGTTCCAGCTTGATAAGGATTAT
ACACTGAATGTCTATAGATTGGCTTTAAAAACTACCTTAAAAAGAGCAAGAAGGAGTATGGAACTTAT
AGCAAGAGAGGATGAAAATCCAAAGGTTGCTGAAGTTATCTATCCAATAATGCAGGTTAATCCTCTTA
ATTATGAGGGCGTTGATGTTGCAGTTGGAGGGATGGAGCAGAGAAAAATACACATGTTAGCAAGGG
AGCTTTTACCAAAAAAGGTTGTTTGTATTCACAACCCTGTCTTAACGGGTTTGGATGGAGAAGGAAAG
ATGAGTTCTTCAAAAGGGAATTTTATAGCTGTTGATGACTCTCCAGAAGAGATTAGGGCTAAGATAAA
GAAAGCATACTGCCCAGCTGGAGTTGTTGAAGGAAATCCAATAATGGAGATAGCTAAATACTTCCTT
GAATATCCTTTAACCATAAAAAGGCCAGAAAAATTTGGTGGAGATTTGACAGTTAATAGCTATGAGGA
GTTAGAGAGTTTATTTAAAAATAAGGAATTGCATCCAATGCGCTTAAAAAATGCTGTAGCTGAAGAAC
TTATAAAGATTTTAGAGCCAATTAGAAAGAGATTATAA

o. DNA sequence of ***p*-IFRS**.

ATGGACGAATTTGAAATGATAAAGAGAAACACATCTGAAATTATCAGCGAGGAAGAGTTAAGAGAGG
TTTTAAAAAAAGATGAAAAATCTGCTCTGATAGGTTTTGAACCAAGTGGTAAAATACATTTAGGGCATT
ATCTCCAAATAAAAAAGATGATTGATTTACAAAATGCTGGATTTGATATAATTATATTGTTGGCTGATTT
ACACGCCTATTTAAACCAGAAAGGAGAGTTGGATGAGATTAGAAAAATAGGAGATTATAACAAAAAAG
TTTTTGAAGCAATGGGGTTAAAGGCAAAATATGTTTATGGAAGTTCGTTCCAGCTTGATAAGGATTAT
ACACTGAATGTCTATAGATTGGCTTTAAAAACTACCTTAAAAAGAGCAAGAAGGAGTATGGAACTTAT
AGCAAGAGAGGATGAAAATCCAAAGGTTGCTGAAGTTATCTATCCAATAATGCAGGTTAATCCTCTTC
ATTATGAGGGCGTTGATGTTGCAGTTGGAGGGATGGAGCAGAGAAAAATACACATGTTAGCAAGGG
AGCTTTTACCAAAAAAGGTTGTTTGTATTCACAACCCTGTCTTAACGGGTTTGGATGGAGAAGGAAAG
ATGAGTTCTTCAAAAGGGAATTTTATAGCTGTTGATGACTCTCCAGAAGAGATTAGGGCTAAGATAAA
GAAAGCATACTGCCCAGCTGGAGTTGTTGAAGGAAATCCAATAATGGAGATAGCTAAATACTTCCTT
GAATATCCTTTAACCATAAAAAGGCCAGAAAAATTTGGTGGAGATTTGACAGTTAATAGCTATGAGGA
GTTAGAGAGTTTATTTAAAAATAAGGAATTGCATCCAATGCGCTTAAAAAATGCTGTAGCTGAAGAAC
TTATAAAGATTTTAGAGCCAATTAGAAAGAGATTATAA

p. DNA sequence of **T7 RNAP**. Codons that were mutated to amber stop codons in the course of developing PACE selections are highlighted in yellow (Ser-12, Ser-203, Tyr-250, Tyr-312, and Ser-527).

ATGAACACGATTAACATCGCTAAGAACGACTTCTCTGACATCGAACTGGCTGCTATCCCGTTCAACAC
TCTGGCTGACCATTACGGTGAGCGTTTAGCTCGCGAACAGTTGGCCCTTGAGCATGAGTCTTACGAG
ATGGGTGAAGCACGCTTCCGCAAGATGTTTGAGCGTCAACTTAAAGCTGGTGAGGTTGCGGATAAC
GCTGCCGCCAAGCCTCTCATCACTACCCTACTCCCTAAGATGATTGCACGCATCAACGACTGGTTTG
AGGAAGTGAAAGCTAAGCGCGGCAAGCGCCCGACAGCCTTCCAGTTCCTGCAAGAAATCAAGCCGG
AAGCCGTAGCGTACATCACCATTAAGACCACTCTGGCTTGCCTAACCAGTGCTGACAATACAACCGT
TCAGGCTGTAGCAAGCGCAATCGGTCGGGCCATTGAGGACGAGGCTCGCTTCGGTCGTATCCGTGA
CCTTGAAGCTAAGCACTTCAAGAAAAACGTTGAGGAACAACTCAACAAGCGCGTAGGGCACGTCTAC
AAGAAAGCATTTATGCAAGTTGTCGAGGCTGACATGCTCTCTAAGGGTCTACTCGGTGGCGAGGCGT
GGTCTTCGTGGCATAAGGAAGACTCTATTCATGTAGGAGTACGCTGCATCGAGATGCTCATTGAGTC
AACCGGAATGGTTAGCTTACACCGCCAAAATGCTGGCGTAGTAGGTCAAGACTCTGAGACTATCGAA
CTCGCACCTGAATACGCTGAGGCTATCGCAACCCGTGCAGGTGCGCTGGCTGGCATCTCTCCGATG
TTCCAACCTTGCGTAGTTCCTCCTAAGCCGTGGACTGGCATTACTGGTGGTGGCTATTGGGCTAACG
GTCGTCGTCCTCTGGCGCTGGTGCGTACTCACAGTAAGAAAGCACTGATGCGCTACGAAGACGTTT
ACATGCCTGAGGTGTACAAAGCGATTAACATTGCGCAAAACACCGCATGGAAAATCAACAAGAAAGT
CCTAGCGGTCGCCAACGTAATCACCAAGTGGAAGCATTGTCCGGTCGAGGACATCCCTGCGATTGA
GCGTGAAGAACTCCCGATGAAACCGGAAGACATCGACATGAATCCTGAGGCTCTCACCGCGTGGAA
ACGTGCTGCCGCTGCTGTGTACCGCAAGGACAAGGCTCGCAAGTCTCGCCGTATCAGCCTTGAGTT
CATGCTTGAGCAAGCCAATAAGTTTGCTAACCATAAGGCCATCTGGTTCCCTTACAACATGGACTGG
CGCGGTCGTGTTTACGCTGTGTCAATGTTCAACCCGCAAGGTAACGATATGACCAAAGGACTGCTTA
CGCTGGCGAAAGGTAAACCAATCGGTAAGGAAGGTTACTACTGGCTGAAAATCCACGGTGCAAACT
GTGCGGGTGTCGATAAGGTTCCGTTCCCTGAGCGCATCAAGTTCATTGAGGAAAACCACGAGAACAT
CATGGCTTGCGCTAAGTCTCCACTGGAGAACACTTGGTGGGCTGAGCAAGATTCTCCGTTCTGCTTC
CTTGCGTTCTGCTTTGAGTACGCTGGGGTACAGCACCACGGCCTGAGCTATAACTGCTCCCTTCCGC
TGGCGTTTGACGGGTCTTGCTCTGGCATCCAGCACTTCTCCGCGATGCTCCGAGATGAGGTAGGTG
GTCGCGCGGTTAACTTGCTTCCTAGTGAAACCGTTCAGGACATCTACGGGATTGTTGCTAAGAAAGT
CAACGAGATTCTACAAGCAGACGCAATCAATGGGACCGATAACGAAGTAGTTACCGTGACCGATGAG
AACACTGGTGAAATCTCTGAGAAAGTCAAGCTGGGCACTAAGGCACTGGCTGGTCAATGGCTGGCT
TACGGTGTTACTCGCAGTGTGACTAAGCGTTCAGTCATGACGCTGGCTTACGGGTCCAAAGAGTTCG
GCTTCCGTCAACAAGTGCTGGAAGATACCATTCAGCCAGCTATTGATTCCGGCAAGGGTCTGATGTT
CACTCAGCCGAATCAGGCTGCTGGATACATGGCTAAGCTGATTTGGGAATCTGTGAGCGTGACGGT
GGTAGCTGCGGTTGAAGCAATGAACTGGCTTAAGTCTGCTGCTAAGCTGCTGGCTGCTGAGGTCAA
AGATAAGAAGACTGGAGAGATTCTTCGCAAGCGTTGCGCTGTGCATTGGGTAACTCCTGATGGTTTC
CCTGTGTGGCAGGAATACAAGAAGCCTATTCAGACGCGCTTGAACCTGATGTTCCTCGGTCAGTTCC
GCTTACAGCCTACCATTAACACCAACAAAGATAGCGAGATTGATGCACACAAACAGGAGTCTGGTAT
CGCTCCTAACTTTGTACACAGCCAAGACGGTAGCCACCTTCGTAAGACTGTAGTGTGGGCACACGA
GAAGTACGGAATCGAATCTTTTGCACTGATTCACGACTCCTTCGGTACCATTCCGGCTGACGCTGCG
AACCTGTTCAAAGCAGTGCGCGAAACTATGGTTGACACATATGAGTCTTGTGATGTACTGGCTGATTT
CTACGACCAGTTCGCTGACCAGTTGCACGAGTCTCAATTGGACAAAATGCCAGCACTTCCGGCTAAA
GGTAACTTGAACCTCCGTGACATCTTAGAGTCGGACTTCGCGTTCGCGTAA

q. DNA sequence of **gene III**. Codons that were mutated to amber stop codons in the course of developing PACE selections are highlighted in yellow (Pro-29, Pro-83, Thr-177, and Tyr-184).

ATGAAAAAATTATTATTCGCAATTCCTTTAGTTGTTCCTTTCTATTCTCACTCCGCTGAAACTGTTGAAA
GTTGTTTAGCAAAACCCCATACAGAAAATTCATTTACTAACGTCTGGAAAGACGACAAAACTTTAGAT
CGTTACGCTAACTATGAGGGCTGTCTGTGGAATGCTACAGGCGTTGTAGTTTGTACTGGTGACGAAA
CTCAGTGTTACGGTACATGGGTTCCTATTGGGCTTGCTATCCCTGAAAATGAGGGTGGTGGCTCTGA
GGGTGGCGGTTCTGAGGGTGGCGGTTCTGAGGGTGGCGGTACTAAACCTCCTGAGTACGGTGATA
CACCTATTCCGGGCTATACTTATATCAACCCTCTCGACGGCACTTATCCGCCTGGTACTGAGCAAAA
CCCCGCTAATCCTAATCCTTCTCTTGAGGAGTCTCAGCCTCTTAATACTTTCATGTTTCAGAATAATAG
GTTCCGAAATAGGCAGGGGGCATTAACTGTTTATACGGGCACTGTTACTCAAGGCACTGACCCCGTT
AAAACTTATTACCAGTACACTCCTGTATCATCAAAAGCCATGTATGACGCTTACTGGAACGGTAAATT
CAGAGACTGCGCTTTCCATTCTGGCTTTAATGAGGATCCATTCGTTTGTGAATATCAAGGCCAATCGT
CTGACCTGCCTCAACCTCCTGTCAATGCTGGCGGCGGCTCTGGTGGTGGTTCTGGTGGCGGCTCTG
AGGGTGGTGGCTCTGAGGGTGGCGGTTCTGAGGGTGGCGGCTCTGAGGGAGGCGGTTCCGGTGG
TGGCTCTGGTTCCGGTGATTTTGATTATGAAAAGATGGCAAACGCTAATAAGGGGGCTATGACCGAA
AATGCCGATGAAAACGCGCTACAGTCTGACGCTAAAGGCAAACTTGATTCTGTCGCTACTGATTACG
GTGCTGCTATCGATGGTTTCATTGGTGACGTTTCCGGCCTTGCTAATGGTAATGGTGCTACTGGTGA
TTTTGCTGGCTCTAATTCCCAAATGGCTCAAGTCGGTGACGGTGATAATTCACCTTTAATGAATAATTT
CCGTCAATATTTACCTTCCCTCCCTCAATCGGTTGAATGTCGCCCTTTTGTCTTTGGCGCTGGTAAAC
CTTACGAGTTCAGTATCGACTGCGATAAGATCAACCTGTTCCGCGGTGTCTTTGCGTTTCTTTTATAT
GTTGCCACCTTTATGTATGTATTTTCTACGTTTGCTAACATACTGCGTAATAAGGAGTCTTAA

r. DNA sequence of **luxAB(Y361TAG)**. The in-frame amber stop codon is highlighted in yellow.

ATGAAATTTGGAAACTTTTTGCTTACATACCAACCTCCCCAATTTTCCCAAACAGAGGTAATGAAACGT
TTGGTTAAATTAGGTCGCATCTCTGAGGAGTGTGGTTTTGATACCGTATGGTTACTGGAGCATCATTT
CACGGAGTTTGGTTTGCTTGGTAACCCTTATGTCGCTGCTGCATATTTACTTGGCGCGACTAAAAAAT
TGAATGTAGGAACTGCCGCTATTGTTCTTCCCACAGCCCATCCAGTACGCCAACTTGAAGATGTGAA
TTTATTGGATCAAATGTCAAAAGGACGATTTCGGTTTGGTATTTGCCGAGGGCTTTACAACAAGGACT
TTCGCGTATTCGGCACAGATATGAATAACAGTCGCGCCTTAGCGGAATGCTGGTACGGGCTGATAAA
GAATGGCATGACAGAGGGATATATGGAAGCTGATAATGAACATATCAAGTTCCATAAGGTAAAAGTAA
ACCCCGCGGCGTATAGCAGAGGTGGCGCACCGGTTTATGTGGTGGCTGAATCAGCTTCGACGACTG
AGTGGGCTGCTCAATTTGGCCTACCGATGATATTAAGTTGGATTATAAATACTAACGAAAAGAAAGCA
CAACTTGAGCTTTATAATGAAGTGGCTCAAGAATATGGGCACGATATTCATAATATCGACCATTGCTT
ATCATATATAACATCTGTAGATCATGACTCAATTAAAGCGAAAGAGATTTGCCGGAAATTTCTGGGGC
ATTGGTATGATTCTTATGTGAATGCTACGACTATTTTTGATGATTCAGACCAAACAAGAGGTTATGATT
TCAATAAAGGGCAGTGGCGTGACTTTGTATTAAAAGGACATAAAGATACTAATCGCCGTATTGATTAC
AGTTACGAAATCAATCCCGTGGGAACGCCGCAGGAATGTATTGACATAATTCAAAAAGACATTGATG
CTACAGGAATATCAAATATTTGTTGTGGATTTGAAGCTAATGGAACAGTAGACGAAATTATTGCTTCC
ATGAAGCTCTTCCAGTCTGATGTCATGCCATTTCTTAAAGAAAAACAACGTTCGCTATTATATTAGGG
CGGTGGCGGTAGCGGCGGTGGCGGTAGCGGCGGTGGCGGTAGCGGCGGTGGCGGTAGCAAATTT
GGATTGTTCTTCCTTAACTTCATCAATTCAACAACTGTTCAAGAACAGAGTATAGTTCGCATGCAGGA
AATAACGGAGTATGTTGATAAGTTGAATTTTGAACAGATTTTAGTGTATGAAAATCATTTTTCAGATAA
TGGTGTTGTCGGCGCTCCTCTGACTGTTTCTGGTTTTCTGCTCGGTTTAACAGAGAAAATTAAAATTG
GTTCATTAAATCACATCATTACAACTCATCATCCTGTCCGCATAGCGGAGGAAGCTTGCTTATTGGAT
CAGTTAAGTGAAGGGAGATTTATTTTAGGGTTTAGTGATTGCGAAAAAAAAGATGAAATGCATTTTTTT
AATCGCCCGGTTGAATATCAACAGCAACTATTTGAAGAGTGTTATGAAATCATTAACGATGCTTTAAC
AACAGGCTATTGTAATCCAGATAACGATTTTTATAGCTTCCCTAAAATATCTGTAAATCCCCATGCTTA
TACGCCAGGCGGACCTCGGAAATATGTAACAGCAACCAGTCATCATATTGTTGAGTGGGCGGCCAA
AAAAGGTATTCCTCTCATCTTTAAGTGGGATGATTCTAATGATGTTAGATATGAATATGCTGAAAGATA
TAAAGCCGTTGCGGATAAATATGACGTTGACCTATCAGAGATAGACCATCAGTTAATGATATTAGTTA
ACTATAACGAAGATAGTAATAAAGCTAAACAAGAGACGCGTGCATTTATTAGTGATTATGTTCTTGAAA
TGCACCCTAATGAAAATTTCGAAAATAAACTTGAAGAAATAATTGCAGAAAACGCTGTCGGAAATTAT
ACGGAGTGTATAACTGCGGCTAAGTTGGCAATTGAAAAGTGTGGTGCGAAAAGTGTATTGCTGTCCT
TTGAACCAATGAATGATTTGATGAGCCAAAAAAATGTAATCAATATTGTTGATGATAATATTAAGAAGT
ACCACACGGAATATACCTAA

s. DNA sequence of **wt sfGFP** expressed from **pET28b(+)-sfGFP**. Codons Asn-39, which was mutated to an amber stop codon in the course of characterizing *Mj*TyrRS variants, is highlighted yellow.

ATGAGCAAGGGCGAAGAACTGTTTACGGGCGTGGTGCCGATTCTGGTGGAACTGGATGGTGATGTC
AATGGTCACAAATTCAGCGTGCGCGGCGAAGGTGAAGGCGATGCAACCAATGGTAAACTGACGCTG
AAGTTTATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTCACCACGCTGACGTAT
GGTGTTCAGTGTTTCAGTCGTTACCCGGATCACATGAAACGCCACGACTTTTTCAAGTCCGCGATGC
CGGAAGGTTATGTCCAAGAACGTACCATCTCATTTAAAGATGACGGCACCTACAAAACGCGCGCCGA
AGTGAAATTCGAAGGTGATACGCTGGTTAACCGTATTGAACTGAAAGGCATCGATTTTAAGGAAGAC
GGTAATATTCTGGGCCATAAACTGGAATATAACTTCAATTCGCACAACGTGTACATCACCGCAGATAA
GCAGAAGAACGGTATCAAGGCTAACTTCAAGATCCGCCATAATGTGGAAGATGGCAGCGTTCAACTG
GCCGACCACTATCAGCAAAACACCCCGATTGGTGATGGCCCGGTCCTGCTGCCGGACAATCATTAC
CTGAGCACGCAGTCTGTGCTGAGTAAAGATCCGAACGAAAAGCGTGACCACATGGTCCTGCTGGAA
TTCGTGACCGCGGCCGGCATCACGCACGGTATGGACGAACTGTATAAAGGCTCACTCGAGCACCAC
CACCACCACCACTGA

t. DNA sequence of **wt sfGFP** expressed from **pBAD-sfGFP**. Codons that were mutated to amber stop codons in the course of characterizing PylRS variants are highlighted in yellow (Ser-2, Asn-39, Asn-135, and Tyr-151).

ATGAGCAAGGGCGAAGAACTGTTTACGGGCGTGGTGCCGATTCTGGTGGAACTGGATGGTGATGTC
AATGGTCACAAATTCAGCGTGCGCGGCGAAGGTGAAGGCGATGCAACCAATGGTAAACTGACGCTG
AAGTTTATTTGCACCACGGGTAAACTGCCGGTTCCGTGGCCGACCCTGGTCACCACGCTGACGTAT
GGTGTTCAGTGTTTCAGTCGTTACCCGGATCACATGAAACGCCACGACTTTTTCAAGTCCGCGATGC
CGGAAGGTTATGTCCAAGAACGTACCATCTCATTTAAAGATGACGGCACCTACAAAACGCGCGCCGA
AGTGAAATTCGAAGGTGATACGCTGGTTAACCGTATTGAACTGAAAGGCATCGATTTTAAGGAAGAC
GGTAATATTCTGGGCCATAAACTGGAATATAACTTCAATTCGCACAACGTGTACATCACCGCAGATAA
GCAGAAGAACGGTATCAAGGCTAACTTCAAGATCCGCCATAATGTGGAAGATGGCAGCGTTCAACTG
GCCGACCACTATCAGCAAAACACCCCGATTGGTGATGGCCCGGTCCTGCTGCCGGACAATCATTAC
CTGAGCACGCAGTCTGTGCTGAGTAAAGATCCGAACGAAAAGCGTGACCACATGGTCCTGCTGGAA
TTCGTGACCGCGGCCGGCATCACGCACGGTATGGACGAACTGTATAAAGGCTCACATCATCATCATC
ATCATTGA

Supplementary References

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2. Ringquist, S. *et al.* Translation initiation in Escherichia coli: sequences within the ribosome-binding site. *Mol. Microbiol.* **6**, 1219-1229 (1992).