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Supplemental Information

A Highly Productive, One-Pot Cell-Free Protein Synthesis Platform Based on Genomically Recoded *Escherichia coli*

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Figure S1. Single lysine mutations are insufficient to confer resistance to OmpT proteolysis. *Related to Figure 4.* Comparison between C321.Δ*A*.759.T7 and mutant strains in which K183 has been mutated to glycine (C321.Δ*A*.759.T7.K183G*)* or leucine (C321.Δ*A*.759.T7.K183L). **(a)** Characterization of the K183 mutants in CFPS. Lysates derived from C321.Δ*A*.759.T7 and the K183 mutant strains were directed to synthesize sfGFP in CFPS both with and without supplementation with purified T7 RNAP, and fluorescence was measured after incubation for 20 hrs at 30°C. Three independent CFPS reactions were performed for each condition, and one standard deviation is shown. **(b)** α-His western blot comparison of the K172 mutant strains.

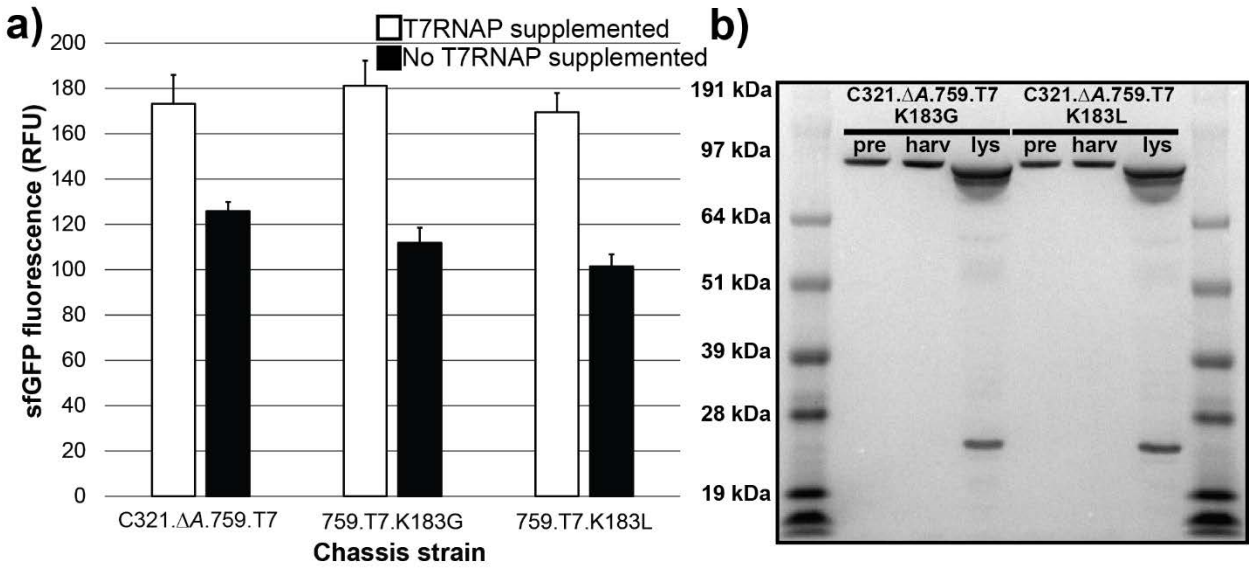

Figure S2. Optimization of orthogonal translation system (OTS) component supplementation for performing amber suppression using 759.T7.Opt lysates. *Related to Figure 6.* 759.T7.Opt lysates were directed to synthesize sfGFP2UAG in CFPS with T7RNAP supplemented. Concentrations of pAcF OTS components (pAcF, **(a)**; pAcFRS, **(b)**; o-tz-tRNA linear expression template (LET)[1], **(c)**) were titrated to identify the optimal concentration of each component. 3 independent reactions were performed per condition, and one standard deviation is shown.

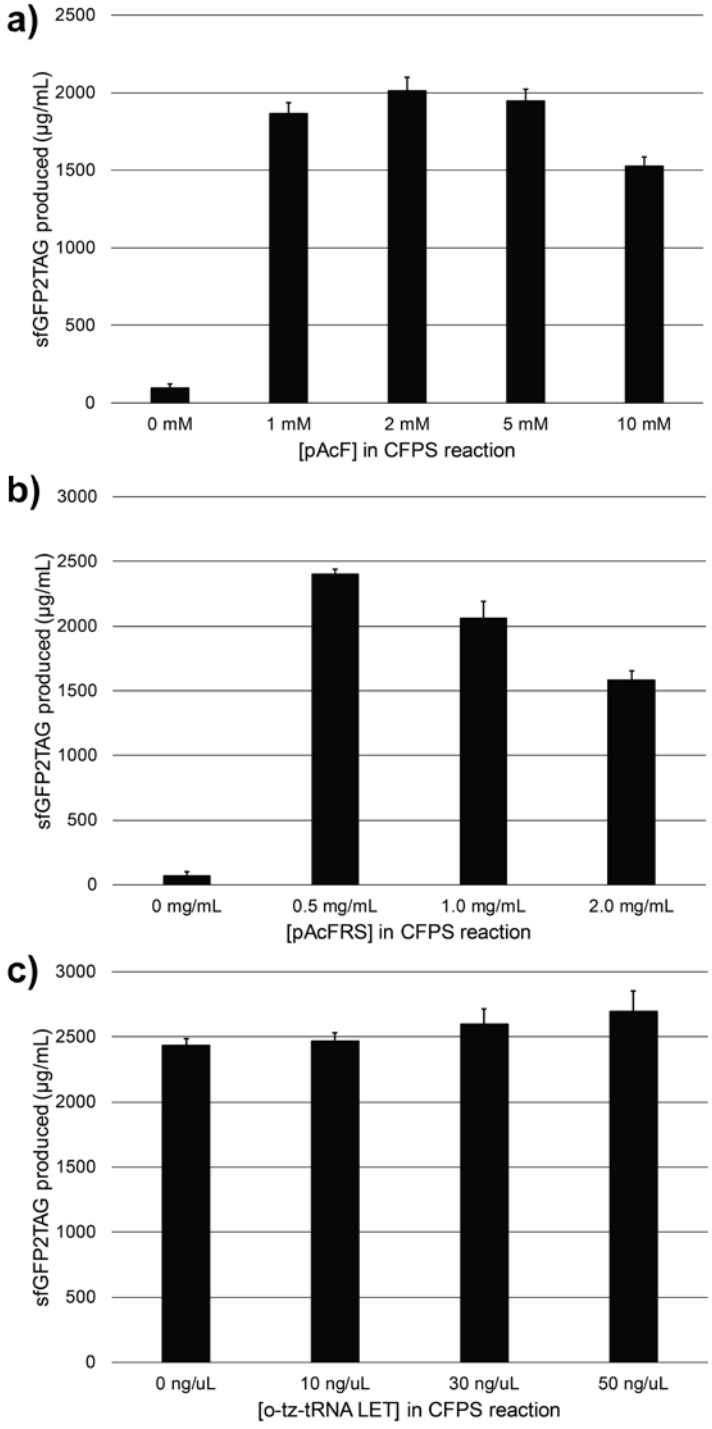

Figure S3. UniDec deconvolution of ELP-40WT spectrum. *Related to Figure 7.* **(a)** the unmodified input spectrum for this construct. Circles designate charge state peaks used by the algorithm to deconvolute the intact mass of the species. **(b)** the deconvoluted mass spectrum generated by the algorithm. The main peak indicated by the circle has an intact mass of 52,478 Da.

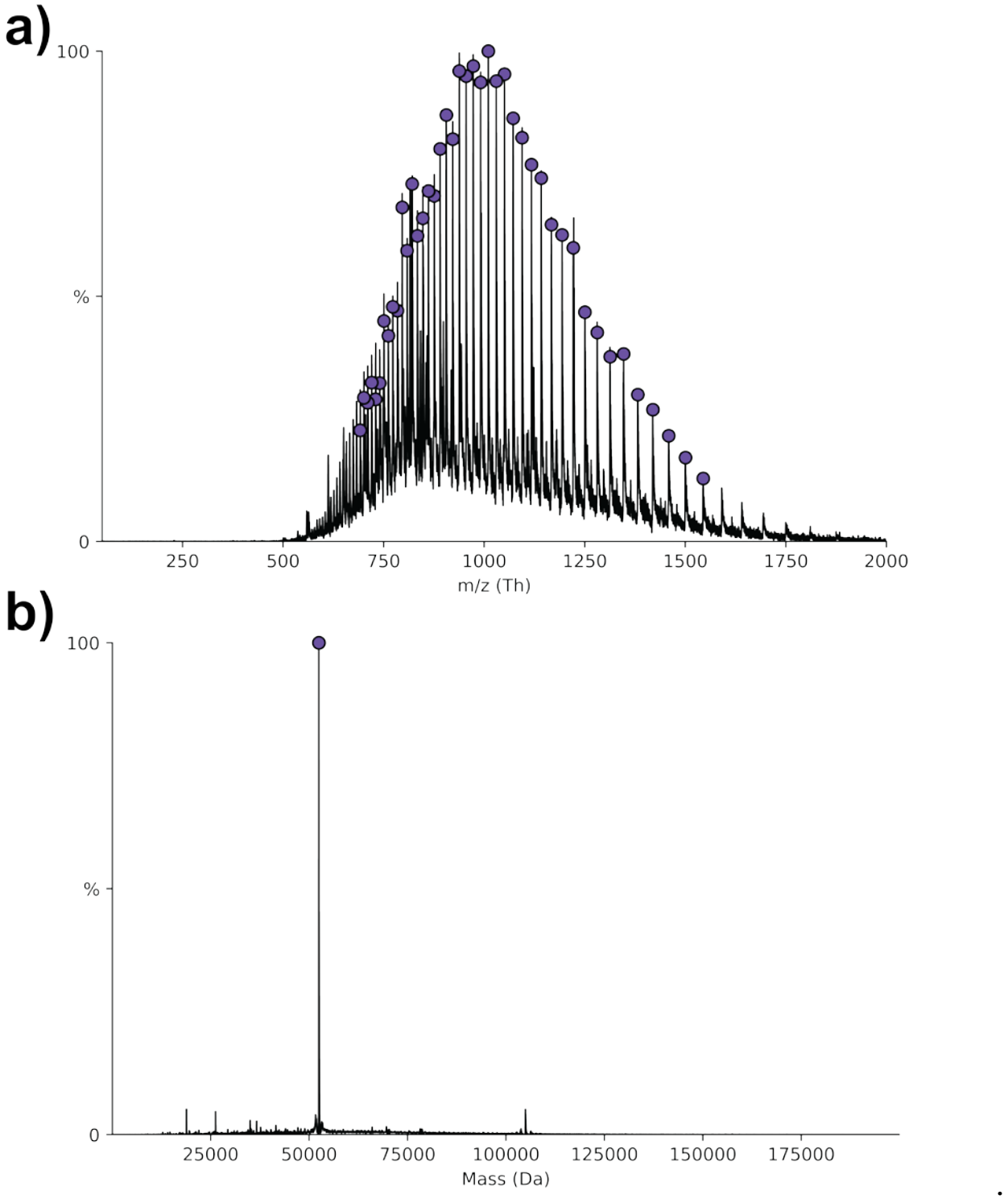

Figure S4. UniDec deconvolution of ELP-40UAG spectrum. *Related to Figure 7.* **(a)** background-subtracted input spectrum for this construct (Subtract curved, value 25.0). Circles designate charge state peaks used by the algorithm to deconvolute the intact mass of the species. **(b)** the deconvoluted mass spectrum generated by the algorithm. The main peak indicated by the circle has an intact mass of 53,540 Da.

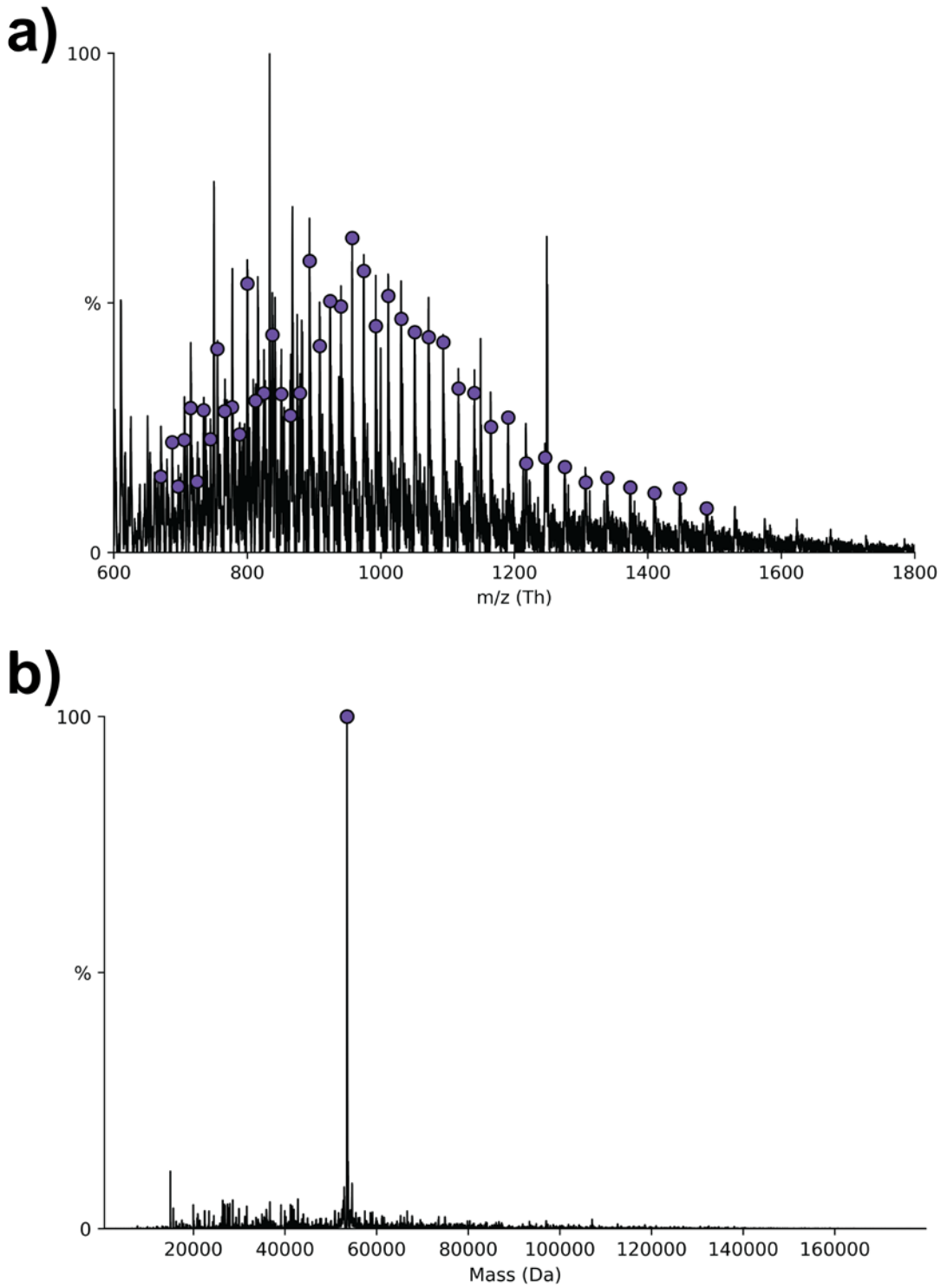

Table S1. Primers used for insert component amplification and assembly, *ompT* knockout, MAGE, colony PCR, T7 plasmid cloning, and DNA sequencing. Underlined bold text indicates location of mismatches. The first four bases of the 5'-MAGE oligonucleotides were phosphorothioated (*). *Related to Figures 2, 4, and 5.*

Primer Name	DNA Sequence (listed 5' to 3')
T7 insert assembly	
lacUV5_asl_F	TGTAGGCTGGATAAGATGCGTCAGCATCGCATCCGGCAAAGGCAGA TCTCGCTTCCGGCTCGTATAATGTGT
PtacI_asl_F	TGTAGGCTGGATAAGATGCGTCAGCATCGCATCCGGCAAAGGCAGA TCTCGAGCTGTTGACAATTAATCATCG
Lpp5_asl_F	TGTAGGCTGGATAAGATGCGTCAGCATCGCATCCGGCAAAGGCAGA TCTCATCAAAAAAATATTGACAAC
asl_homology_R	AATATCCACCACGCGCGCAGATTAAATCTGACTAAGCCGGCGCTATC GCTGGTGGAATCGAAATCTCGTGATGG
asl_F	TGTAGGCTGGATAAGATGC
asl_R	AATATCCACCACGCGCGCAG
lacUV5_int_F	TGCTTCTCATAGAGTCTTGCAGACAAACTGCGCAACTCGTGAAAGGT AGGGCTTCCGGCTCGTATAATGTGT
PtacI_int_F	TGCTTCTCATAGAGTCTTGCAGACAAACTGCGCAACTCGTGAAAGGT AGGGAGCTGTTGACAATTAATCATCG
Lpp5_int_F	TGCTTCTCATAGAGTCTTGCAGACAAACTGCGCAACTCGTGAAAGGT AGGATCAAAAAAATATTGACAAC
int_homology_R	ATTTTATGCGCGCACGAAAAGCATCAGGTCTTTCCTTCGAAGGGGAT CCGGGTGGAATCGAAATCTCGTGATGG
int_F	TGCTTCTCATAGAGTCTTGC
int_R	ATTTTATGCGCGCACGAAAAG
lacUV5_promRBS_R	CGATCCTCTCATTTTGTACCTCCTTAGTTGCTTGCAATTGTTATCCGC TCACAATTCC
lacUV5_RBST7_F	CAAAATGAGAGGATCGCATCACCATCACCATCACGGATCCAACACG ATTAACATCGCTAA
Lpp5_promRBS_R	GATCCTCTCATTATGTACCTCCTTACTGTTTTGTTTTAATTGTTATCCG CTCACAATTCC
Lpp5_RBST7_F	CATAATGAGAGGATCGCATCACCATCACCATCACGGATCCAACACG ATTAACATCGCTAA
PtacI_promRBS_R	ATCCTCTCATATATTACCTCCTTAGTAGCGCTGTGTGTAATTGTTATC CGCTCACAATTC
PtacI_RBST7_F	ATATATGAGAGGATCGCATCACCATCACCATCACGGATCCAACACG ATTAACATCGCTAA
T7_synterm_R	CCTGTATCAGGCTGAAAATCTTACGCGAACGCGAAGTCCGACTC
synterm_F	GGACTTCGCGTTCGCGTAAGATTTTCAGCCTGATACAGG
Synterm_kanR_R	CTTTCTACGTGTTCCGCTTATAAAGTGTAAAGCCTGG
kanR_F	AAGCGGAACACGTAGAAAG
Synthetic_term (L3S2P21)	GATTTTCAGCCTGATACAGGATTTTCAGCCTGATACAGCTCGGTACC AAATTCCAGAAAAGAGGCCTCCCGAAAGGGGGGCCTTTTTTCGTTTT GGTCCCCTTTTTGCGTTTCTACACCCAGGCTTTACACTTTAT
***ompT* knockout**	
delompT_ F	CGACTACATCCGTGAGGTGAATGTGGTGAAGTCTGCCCGTGTCGGTT ATTGAAGCGGAACACGTAGAAAG

delompT_ R	TAATGGTAAAAAGCTGTCACAATTCATAAAAAACCTTAATATACGCC ACCGGTGGAATCGAAATCTCGTGATG
MAGE	
MAGE_T7_K172L	T*C*G*A*CAACTTGCATAAATGCTTTCTTGTAGACGTGCCCTACGCG **CAG**GTTGAGTTGTTCCTCAACGTTTTTCTTGAAGTGCTTAGCTTCA
MAGE_T7_K172G	T*C*G*A*CAACTTGCATAAATGCTTTCTTGTAGACGTGCCCTACGCG **GCC**GTTGAGTTGTTCCTCAACGTTTTTCTTGAAGTGCTTAGCTTCA
MAGE_T7_double	C*A*G*C*CTCGACAACTTGCATAAATGCTTT**CGC**GTAGACGTGCCCT ACGCG**GCC**GTTGAGTTGTTCCTCAACGTTTTTCTTGAAGTGCTTAG
delkanR_oligo	G*G*T*T*GGGCGTCGCTTGGTCGGTCATTTCGAACCCCAGAGTCCCG CCATGCGAAACGATCCTCATCCTGTCTCTTGATCAGATCTTGATCC
delampR_oligo	T*T*T*G*CCGACTACCTTGGTGATCTCGCCTTTCACGTAGTGGACAA AACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATGT
T7delHis_MAGE	c*a*g*t*tcgatgtcagagaagtcgttcttagcgatgttaatcgtgttCATTATGTACCTCCTTAC TGTTTTGTTTTAATTGTTATCCGCTCA
Colony PCR	
K172L_cPCRF_mut	CGTTGAGGAACAACTCAACCTG
K172G_cPCRF_mut	CGTTGAGGAACAACTCAACGG
K172_cPCRF_wt	CGTTGAGGAACAACTCAACAA
172mut_cPCR_R	TTGTTGATTTTCCATGCGGTG
K179A_cPCRF_mut	CGCGTAGGGCACGTCTACGC
K179_cPCRF_wt	CGCGTAGGGCACGTCTACAA
179mut_cPCR_R	TTGGCGACCGCTAGGACTTTC
pUC_T7_cPCRF	GCGATAAGTCGTGTCTTACC
pUC_T7_cPCRR	CATGTAAACGTCTTCGTAGC
delompT_cPCR1_F	GGGACTATTGAGTACGAACG
delompT_cPCR2_R	CGAATCTCATAACGCAAACC
T7delHisMASC_His	GCATCACCATCACCATCACG
T7delHisMASC_wtF	GGAGGTACATAATGaacacg
T7delHisMASC_R	tttccatgcggtgttttgcg
cureALL_cPCR_F	ggcgataagtcgtgtcttac
cureALL_cPCR_R	ttgccatcctatggaactgc
T7 plasmid cloning	
pUC_T7asl_F	CTGCGCGCGTGGTGGATATTGCATGCATCTCCTCAGATTGATTTAAA
pUC_T7asl_R	CGCATCTTATCCAGCCTACAGCATGCATCTCCTCGCTCACTGACTCG CTG
pUC_T7int_F	TTTTCGTGCGCGCATAAAATGCATGCATCTCCTCAGATTGATTTAAA ACTTCAT
pUC_T7int_R	GCAAGACTCTATGAGAAGCAGCATGCATCTCCTCGCTCACTGACTCG CTG
p15a_T7asl_F	CTGCGCGCGTGGTGGATATTGCATGCATCTCCTCTCCCTTAACGTGA GTTTTCG
p15a_T7asl_R	CGCATCTTATCCAGCCTACAGCATGCATCTCCTCTGAGTCAGCAACA CC

DNA Sequencing

T7_asl_seq1_F	GGCCGCCTGCGGTTGATTGC
T7_asl_seq_R	GTGTGGACCAGACATCCTTC
T7_int_seq1_F	TTCAATTTTGTCCCACTCCCTGC
T7_int_seq_R	ACGAATACCTGAAAATTTATCAAGCAGC
T7_seq2_F	AGCCGGAAGCCGTAGCGTAC
T7_seq3_F	ACGTTTACATGCCTGAGGTG
T7_seq4_F	GCTTCCTTGCGTTCTGCTTTG
T7_seq5_F	TCAAAGATAAGAAGACTGGAG
T7_seq6_F	GGGGCCTTTTTTCGTTTTGG
T7_seq7_F	GCAGCTGTGCTCGACGTTGTC
pMAZ_seq	caattcagcaaattgtgaacatcatc

pMAZ plasmid assembly

T7delHis_oligo1	gagcacTAATCGTGTTGGATCCGTGAgttttagagctagaaat
T7delHis_oligo2	ctaaaacTCACGGATCCAACACGATTAgtgctcagtatctct
pMAZbb_F	AGCTAGAAAUAGCAAGTTAAAATAAGGC
pMAZbb_R	AGTATCTCUATCACTGATAGGGATGTCA
pMAZCurebb_F	gagaagcacacggtcacac
pMAZCurebb_R	ttgccatcctatggaactgc

Table S2. Strains and plasmids used in this study. Km^R, Ap^R, and Cm^R are kanamycin, ampicillin, and chloramphenicol resistance, respectively. 'Δ' indicates deleted gene/feature, and '∇' indicates gene(s) inserted into the genome at the specified locus behind the specified promoter. '{}' denote amino acid substitution mutations made in the *1* gene open reading frame. *Related to Figures 2, 3, 4, 5, 6, and 7.*

Strains and plasmids	Genotype/relevant characteristics	Source
Strains		
BL21 Star™ (DE3)	F^- *ompT hsdS*$_B$ ($r_B^- m_B^-$) *gal dcm rne131* (DE3)	ThermoFisher
C321.ΔA.759	*C321.ΔA. endA⁻ gor⁻ rne⁻ mazF⁻*, Ap^R	Martin et al., 2018.
DH5α	F^- *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20* φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17($r_K^- m_K^+$), λ^-	ThermoFisher
759.T7.int.lacUV5	*C321.ΔA.759.∇1.int.lacUV5*, Km^R	This study
759.T7.int.PtacI	*C321.ΔA.759.∇1.int.PtacI*, Km^R	This study
759.T7.int.Lpp5	*C321.ΔA.759.∇1.int.Lpp5*, Km^R	This study
759.T7.asl.lacUV5	*C321.ΔA.759.∇1.asl.lacUV5*, Km^R	This study
759.T7.asl.PtacI	*C321.ΔA.759.∇1.asl.PtacI*, Km^R	This study
759.T7.asl.Lpp5	*C321.ΔA.759.∇1.asl.Lpp5*, Km^R	This study
759.T7.ΔkanR	*C321.ΔA.759.∇1.asl.Lpp5.ΔKm^R*	This study
759.T7.ΔompT	*C321.ΔA.759.∇1.asl.Lpp5.ΔompT*, Km^R	This study
759.T7.K172L	*C321.ΔA.759.∇1.asl.Lpp5{K172L}*, Km^R	This study
759.T7.K172G	*C321.ΔA.759.∇1.asl.Lpp5{K172G}*, Km^R	This study
759.T7.D	*C321.ΔA.759.∇1.asl.Lpp5{K172G, K179A}*, Km^R	This study
759.T7.D.ΔAbR	*C321.ΔA.759.∇1.asl.Lpp5{K172G, K179A}.ΔKm^R. ΔAp^R*	This study
759.T7.Opt	*C321.ΔA.759.∇1.asl.Lpp5{K172G, K179A, ΔHis}.ΔKm^R. ΔAp^R*	This study
Plasmids		
pKD4	Km^R	Datsenko & Wanner, 2000.
pAR1219	Ap^R	Davanloo et al., 1984.
pDPtacIAcRSTT1	Km^R	de Boer et al., 1983.
pDTT1-Lpp5-EF-Tu	Km^R	Gan et al.,
pY71-sfGFP	Km^R, P_{T7}::super folder green fluorescent protein (sfGFP), C-terminal strep-tag	Bundy & Swartz, 2010.
pY71-sfGFP-T216amb	pY71-sfGFP with amber codon at T216	Bundy & Swartz, 2010.
pY71-sfGFP-2amb	pY71-sfGFP with amber codon at N212 and T216	Hong et al., 2014b.

pY71-sfGFP-5amb	pY71-sfGFP with amber codon at D36, K101, E132, D190, and E213	Hong et al., 2014b.
pY71-pAcFRS	P_{T7}::pAcFRS, C-terminal 6x histidine tag	Hong et al., 2014b.
pEVOL-pAcF	Cm^R, P_{glnS}::*pAcFRS*, P_{araBAD}::*pAcFRS*, P_{proK}::o-tRNA	Young et al., 2010.
pY71-T7-tz-o-tRNA	P_{T7}:: hammer-head ribozyme (tz), o-$tRNA^{opt}$ (o-tz-tRNA)	Hong et al., 2014b.
pUC-T7-*int.lacUV5*	Km^R, P_{lacUV5}::T7 RNAP w/*int* locus flanking homology	This study
pUC-T7-*asl.lacUV5*	Km^R, P_{lacUV5}::T7 RNAP w/*asl* locus flanking homology	This study
pUC-T7-*asl.PtacI*	Km^R, P_{PtacI}::T7 RNAP w/*asl* locus flanking homology	This study
pUC-T7-*asl.Lpp5*	Km^R, P_{Lpp5}::T7 RNAP w/*asl* locus flanking homology	This study
P15a-T7-*asl.Lpp5*	Km^R, P_{Lpp5}::T7 RNAP w/*asl* locus flanking homology	This study
P15a-T7-*asl.PtacI*	Km^R, P_{PtacI}::T7 RNAP w/*asl* locus flanking homology	This study
P15a-T7-*int.Lpp5*	Km^R, P_{Lpp5}::T7 RNAP w/*int* locus flanking homology	This study
pMA7CR_2.0	Ap^R, *Tet*::Cas9, *ARA*::λβ,*dam*	Ronda et al., 2016.
pMAZ-SK	Km^R, *Tet::*gRNA	Ronda et al., 2016.
pMAZ-ΔHis	Km^R, *Tet::* Δ*His* gRNA	This study
pMAZ-Cure	Km^R, *Tet::* pMA7CR_2.0 gRNA	This study
pY71-FI-ELP20	FI-ELP-20mer	Martin et al., 2018.
pY71-FI-ELP30	FI-ELP-30mer	Martin et al., 2018.
pY71-FI-ELP40	FI-ELP-40mer	Martin et al., 2018.
pY71-FI-ELP20X	FI-ELP-20mer with 20 amber sites	Martin et al., 2018.
pY71-FI-ELP30X	FI-ELP-30mer with 30 amber sites	Martin et al., 2018.
pY71-FI-ELP40X	FI-ELP-40mer with 40 amber sites	Martin et al., 2018.