Appendix

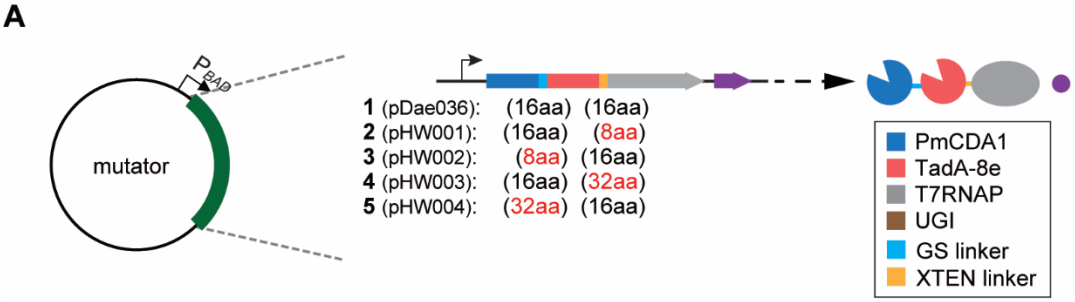
A dual gene-specific mutator system installs all transition mutations at similar rates *in vivo*

Daeje Seo, Ga-eul Eom, Hye Won Kim, and Seokhee Kim*

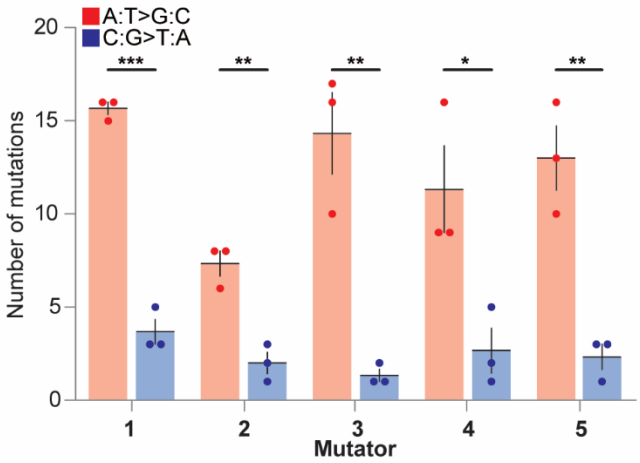
Seoul National University

*Corresponding author: Seokhee Kim, seokheekim@snu.ac.kr

A



B



C

Mutator-clone#	A:T>G:C	mut#	C:G>T:A	mut#	total#
1-1	-131*, -111*, -109*, -93*, -92*, -64*, -17*, D25D, Y31H, H148R, T162T, T170A, E210G, T251A, S314S	15	-33*, Q41X, T216T	3	18
1-2	-98*, -92*, -65*, -64*, -30*, -17*, -1*, E6E, S10G, V21V, D25G(GAT to GGC), Y31R(TAT to CGT), Y198C, K265K	16	-71*, F140F, F158F	3	19
1-3	-92*, -59*, -17*, S2P, N26D, T39A, M42T, A56A, T112A, D114G, I145V, F160L, V173V, R186C, N233S, K324K	16	A5V, I60I, P150S, R186C, P277L	5	21
2-1	-92*, -63*, -17*, D25D, V109V, V173V, E236G, +56*	8	-70*, -36*, V29V	3	11
2-2	-108*, -92*, -59*, G121G, I145T, F220F, +20*, +117*	8	A9A, T251T	2	10
2-3	-59*, -17*, N61D, L83P, Y137C, +23*	6	T251T	1	7
3-1	-133*, -93*, -92*, -85*, -66*, -59*, -44*, -17*, N26D, H37R, I145V, T157A, V173A, Q182R, N233S, Y290H	16	L4L, T126T	2	18
3-2	-98*, -92*, -17*, H3H, I15I, N26D, N72N, F125F, T216A, V269A	10	-71*	1	11
3-3	-92*, -89*, -82*, -81*, -66*, -64*, -59*, -17*, V8V, S16G, G33G, R74R, N84N, I113V, R167R, V173A, I218V	17	G271S	1	18
4-1	-131*, -54*, -17*, D20D, R11R, E117E, F125F, T126A, Y137H	9	P49L	1	10
4-2	-109*, -92*, -77*, -65*, -17*, -6*, -2*, -1*, M1A(ATG to GCG), V21V, D25D, H37R, T91A, Y137C, T168A	16	-110*, N61N, R176C, Q181X, P277L	5	21
4-3	-131*, -17*, K34E, Y198C, Y247C, T251A, K262E, S314S, +23*	9	-71*, L231L	2	11
5-1	-98*, -95*, -83*, -82*, -66*, -59*, -17*, L24L, N26N, Y31C, A82A, H138R, I145A(ATT to GCT), T168A, H228R	16	Q69X	1	17
5-2	-131*, -98*, -96*, -77*, -51*, -17*, G33G, L38L, T112A, T126A, T163A, T177A, I285V	13	-68*, Q41X, A58A	3	16
5-3	-131*, -98*, -93*, -65*, -60*, -17*, -2*, I145V, H148R, V279A	10	-48*, M42I, Q182X	3	13

Amino acids were numbered according to the protein sequence of *pheS*_A294G
A>G mutation on the codoing strand, **red**; **T>C** mutation on the coding strand, **orange**; **C>T** mutation on the coding strand , **blue**; **G>A** mutation on the coding strand, **cyan**
* mutations not made on *pheS*_A294G gene orf but made between a T7 promoter and a T7 terminator

Appendix Figure S1. Linker optimization of a triple fusion protein, PmCDA1_TadA-8e_T7RNAP.

A Design of variants with different linkers: two original linkers (**1**), a shorter linker between TadA-8e and T7RNAP (**2**), a shorter linker between PmCDA1 and TadA-8e (**3**), a longer linker between TadA-8e and T7RNAP (**4**), a longer linker between PmCDA1 and TadA-8e (**5**).

B Number of mutations found in six clones from samples shown in (A) at 20 mutagenesis cycle.

C A list of mutations found in samples shown in (B).

Data are presented as dot plots with mean ± standard deviation (SD) (n = 6). *P < 0.05, **P < 0.01, ***P < 0.001; by Student's t-test.

Appendix Table S1. *E. coli* strains used in this study

Strain	Description	Reference
W3110		
cHYO057	W3110 *Δung::kan*^{R}	(Park & Kim, 2021)
cDJ085	W3110 *ΔalkA::Sm*^{R} *Δnfi::Kan*^{R}	This study

Appendix Table S2. Plasmids used in this study

Plasmid	Construct	Description	reference
pBAD33		Experimental control/cloning vector	
pHyo094	pBAD33-PmCDA1-T7RNAP, ugi	eMutaT7^{PmCDA1}, source of PmCDA1	(Park & Kim, 2021)
pHyo182	pVS133-lacI, pheS_A294G	Target plasmid (pheS_A294G)	(Park & Kim, 2021)
pHyo183	pBAD33-T7RNAP, ugi	control	(Park & Kim, 2021)
pHyo245	pVS133-dualT7_pheS_A294G	Dual promoter system/cloning vector	(Park & Kim, 2021)
pREMCM3		Tetracycline resistance gene	(Melancon & Schultz, 2009)
TadA_mut	BamH1-GS-TadA*-GS-EcoR1	Source of TadA-7.10 gene	(Gaudelli *et al*, 2017)
pDae027	pCDF-TadA-8e-TadA-8e	Source of TadA-8e gene	(Richter *et al*, 2020)
pDae014	pBAD33-PmCDA1-T7RNAP	eMutaT7^{PmCDA1} without *ugi*	This study
pDae028	pBAD33-TadA-7.10-T7RNAP	eMutaT7$^{TadA-7.10}$	This study
pDae029	pBAD33-TadA-8e-T7RNAP	eMutaT7$^{TadA-8e}$	This study
pDae032	pBAD33-TadA-8e	Control	This study
pDae035	pBAD33-TadA-8e-T7RNAP, PmCDA1-T7RNAP, ugi	the expression of two mutators, eMutaT7$^{TadA-8e}$ and eMutaT7^{PmCDA1}	This study
pDae036	pBAD33-PmCDA1-TadA-8e-T7RNAP, ugi	triply-fused protein of PmCDA1, TadA-8e, and T7RNAP	This study
pDae037	pBAD33-TadA-8e-PmCDA1-T7RNAP, ugi	triply-fused protein of TadA-8e, PmCDA1 and T7RNAP	This study
pDae043	pBAD33-PmCDA1-T7RNAP, J23100(promoter), ugi	Constitutive promoter for *ugi*	This study
pDae044	pBAD33-ugi-PmCDA1-T7RNAP	triply-fused protein of *ugi*, PmCDA1, and T7RNAP	This study

pDae069	pBAD33-PmCDA1-T7RNAP, (RBS) ugi	eMutaT7^{PmCDA1} with optimized RBS	This study
pDae079	pBAD33-TadA-8e-T7RNAP, PmCDA1-T7RNAP, (RBS) ugi	eMutaT7^{transition}	This study
pDae080	pBAD33-PmCDA1-T7RNAP, (RBS) ugi, TadA-8e-T7RNAP	the expression of two mutators, eMutaT7^{PmCDA1} and eMutaT7$^{TadA-8e}$ and optimized *ugi*	This study
pHW001	pBAD33-PmCDA1-16aa-TadA-8e-8aa-T7RNAP, ugi	Shoter XTEN linker	This study
pHW002	pBAD33-PmCDA1-8aa-TadA-8e-16aa-T7RNAP, ugi	Shoter GS linker	This study
pHW003	pBAD33-PmCDA1-16aa-TadA-8e-32aa-T7RNAP, ugi	Longer XTEN linker	This study
pHW004	pBAD33-PmCDA1-32aa-TadA-8e-16aa-T7RNAP, ugi	Longer XTEN linker	This study
pGE158	pVS133-dualT7_ss-TEM-1, tetR	Evolution target (TEM-1)	This study

Appendix Table S3. Primers used in this study

Oligonucleotides	Sequence (5'→3')	Description
T7promoter	TAATACGACTCACTATAGGG	Universal sequencing primer
T7terminator	GCTAGTTATTGCTCAGCGG	Universal sequencing primer
pBAD-F	ATGCCATAGCATTTTTATCCA	Universal sequencing primer
pBAD-R	GATTTAATCTGTATCAGG	Universal sequencing primer
022_PxUgT_PxT_ovlp_fw	aacacgattaacatcgctaagaacg	Cloning of TadA-8e-T7RNAP(pDae029)
029_pBAD_Gibson_rv	CCATGGtgaattcctcctGagctcg	Cloning of TadA-7.10-T7RNAP(pDae028)
052_T7RNAPQ265_rv	gttgacgctcaaacatcttgc	
053_PmCDA_P57_rv	gggcttgttgacggcatag	
054_PmCDA_G137_fw	ggactctggaatctgaggg	
055_UPT-GS_ovlp_fw	GTAGCGGCTCTGGTTCCGGCTCTGGTAGCGGATCCAcagacgccgagtacgtg	Clonging of pBAD33-TadA-8e-PmCDA1-T7RNAP, ugi (pDae037)
058_PUT2_GS_ovlp_rv	CCGGAACCAGAGCCGCTACCAGAGCCGGAACCaacggctggagacttagtgg	Clonging of pBAD33-PmCDA1-TadA-8e-T7RNAP, ugi (pDae036)
062_pYH103_ovlp1_rv	ATGCCATGGtgaattcctc	Clonging of pBAD33-TadA-8e-PmCDA1-T7RNAP, ugi (pDae037)
063_PmCDA_ovlp1_fw	ctCaggaggaattcaCCATGGC	Clonging of pBAD33-TadA-8e-PmCDA1-T7RNAP, ugi (pDae037)
128_pYH103_dpoII_rv	catATGCCATGGtgaattcctc	Cloning of TadA-8e-T7RNAP(pDae029)
129_nfi_fw	GGTCACGGCATTCATCAGG	
130_nfi_rv	GACATGCTGCCAGCTTTCC	
131_alkA_fw	GCGAAATGTTGCCGTCGC	
132_alkA_rv	CCCATCGCCTGATGCGAC	
133_TadA8e_IVA_fw	ctCaggaggaattcaCCATGGCATATGAGTGAAGTTGAATTCAGCCATG	Cloning of TadA-8e-T7RNAP(pDae029)
134_TadA8e_IVA_rv	gaagtcgttcttagcgatgttaatcgtgttactttcgggtgtggcg	Cloning of TadA-8e-T7RNAP(pDae029)
137_TadAdimer_del_fw	GGTTCCGGTAGCTTGTCTGAAGTC	Cloning of TadA-7.10-T7RNAP(pDae028)
138_TadA_del_fw	CATATGTCTGAAGTCGAATTTAGCCACG	Cloning of TadA-7.10-T7RNAP(pDae028)
161_dalkA_SmR3_fw	ATGGCGGCAAAATTGACCGCCAGAGTGGCACAGCTTTATGgcgaccgagtgagctagctatttg	Amplification of streptomycin resistance gene from pCDF for construction of alkA k/o strain
162_dalkA_SmR3_rv	GGGAAGCAGATATACTCCGGAAAATCATCCAGCCGTTCGCgaacgaattgttagacattatttgcc	Amplification of streptomycin resistance gene from pCDF for construction of alkA k/o strain

163_Am7(8e)_dL_rv	ggagtctcgctgccgcttaATTAATG CTG	Clonging of TadA-8e(pDae032)
165_Am7(8e)_dT7_fw	ggacttcgcgttcgcgtaa	Clonging of TadA-8e(pDae032)
170_duet_Am7_IVA2_fw	cagaatttgcctggcggcagacttttcata ctcccgccattcagagaag	Clonging of pDae035, pDae079 (=eMutaT7^{transition}), and pDae080
171_duet_tm_Am7_IVA1_rv	cagggttattgtctcatgagcg	Clonging of pDae035, pDae079 (=eMutaT7^{transition}), and pDae080
172_duet_PxT_IVA2_rv	ctgccgccaggcaaattc	Clonging of pDae035, pDae079 (=eMutaT7^{transition}), and pDae080
173_duet_tm_PxT_IVA1_fw	gtatccgctcatgagacaataacc	Clonging of pDae035, pDae079 (=eMutaT7^{transition}), and pDae080
174_PgAxT_PxT_IVA1_fw	ctcccgggacctcagagtc	Clonging of pBAD33-PmCDA1-TadA-8e-T7RNAP, ugi (pDae036)
175_PgAxT_TadA8_IVA1_fw	GTAGCGGCTCTGGTTCCGG CTCTGGTAGCGGATCCAGT GAAGTTGAATTCAGCCATG	Clonging of pBAD33-PmCDA1-TadA-8e-T7RNAP, ugi (pDae036)
176_PgAxT_TadA8_IVA1_rv	ggactctgaggtcccggg	Clonging of pBAD33-PmCDA1-TadA-8e-T7RNAP, ugi (pDae036)
177_AgPxT_TadA8_IVA2_rv	CCGGAACCAGAGCCGCTAC CAGAGCCGGAACCATTAAT GCTGCTCTGTGCTTTCT	Clonging of pBAD33-TadA-8e-PmCDA1-T7RNAP, ugi (pDae037)
182_dnfi_KanR_fw	ATGGATCTCGCGTCATTACG CGCTCAACAAATCGAACTG GCTTGATCCTTTGATCTTTT CTACGGGGtc	Amplification of kanamycin resistance gene from pET28b for construction of nfi k/o strain
183_dnfi_KanR_rv	TTAGGGCTGATTTGCTGTAT AGCGCACGAACGCCGGACG TTCCGATGGCACTTTTCGG GGAAATGTG	Amplification of kanamycin resistance gene from pET28b for construction of nfi k/o strain
184_ugi-F	caaaccctgggctctggtg	
185_Amp-R	cagcatcttttactttcaccagc	
186_T7RNAP_PstI_rv	atactgcagttacgcgaacgcgaagtcc	Clonging of pBAD33-PmCDA1-T7RNAP (pDae014)
192_ugi_IVA-F	ctCaggaggaattcaCCATGGCAT atgaccaacctttccgacatc	Cloning of pBAD33-ugi-PmCDA1-T7RNAP (pDae044)
193_ugi_IVA-R	CCGGAACCAGAGCCGCTAC CAGAGCCGGAACCtagcatcttg atcttgttctctcc	Cloning of pBAD33-ugi-PmCDA1-T7RNAP (pDae044)

194_ugi-J23100-R	ctaggactgagctagccgtcaaaggatcccgggctgcaG	Cloning of pBAD33-PmCDA1-T7RNAP, J23100(promoter), ugi (pDae043)
195_ugi-J23100-RBS-F	gtacagtgctagcctagagtcaggaggagacctgcgatgaccaacctttcc	Cloning of pBAD33-PmCDA1-T7RNAP, J23100(promoter), ugi (pDae043)
223-pHyo250-IVA-F	AGCACCACCACCACCACCACTG	Cloning of pVS133-dualT7_ss-TEM-1, tetR (pGE158)
224-pHyo250-IVA-R	CATGGTATATCTCCTTCTTAAAGTTAAACAAAA	Cloning of pVS133-dualT7_ss-TEM-1, tetR (pGE158)
225-TEM1-IVA-F	CCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACCatgagtattcaacatttccgtgtcg	Cloning of pVS133-dualT7_ss-TEM-1, tetR (pGE158)
226-TEM1-IVA-R	CAGTGGTGGTGGTGGTGGTGCTctcgaGttaccaatgcttaatcagtgagg	Cloning of pVS133-dualT7_ss-TEM-1, tetR (pGE158)
231-AxT-32aa-F	CTCTGGTTCCGGCTCTGGTAGCGGATCCAgcggcagcgagactccc	Cloning of pBAD33-PmCDA1-16aa-TadA-8e-32aa-T7RNAP, ugi (pHW003)
232-AxT-32aa-R	CCGCTACCAGAGCCGGAACCATTAATGCTGCTCTGTGCTTTCTTTTGTG	Cloning of pBAD33-PmCDA1-16aa-TadA-8e-32aa-T7RNAP, ugi (pHW003)
235-GS-32aa-F	ctcagagtccgccacacccgaaagtAGTGAAGTTGAATTCAGCCATG	Cloning of pBAD33-PmCDA1-32aa-TadA-8e-16aa-T7RNAP, ugi (pHW004)
236-GS-32aa-R	gtcccgggagtctcgctgccAcTGGATCCGCTACCA	Cloning of pBAD33-PmCDA1-32aa-TadA-8e-16aa-T7RNAP, ugi (pHW004)
239_PstI_pBAD_fw	ataaCTGCAGgcatgcaagc	Clonging of pBAD33-TadA-7.10-T7RNAP
240-Ftet-IVA-F	ctgtcagaccaagtttactcaactg	Cloning of pVS133-dualT7_ss-TEM-1, tetR (pGE158)
241-Ftet-IVA-R	gacataagtccatcagttcaacgg	Cloning of pVS133-dualT7_ss-TEM-1, tetR (pGE158)
242-TcR-IVA-F	gacttccgttgaactgatggacttatgtcgtaattctcatgtttgacagcttatcatc	Cloning of pVS133-dualT7_ss-TEM-1, tetR (pGE158)
243-TcR-IVA-R	cgcagttgagtaaacttggtctgacagtggagtggtgaatccgttagc	Cloning of pVS133-dualT7_ss-TEM-1, tetR (pGE158)

245-G8aa-F	ccgaaagtAGTGAAGTTGAATTCAGCCATGAATATTG	Cloning of pBAD33-PmCDA1-8aa-TadA-8e-16aa-T7RNAP, ugi (pHW002)
246-G8aa-R	gtgtggcggactctgaaacggctggagacttagtgg	Cloning of pBAD33-PmCDA1-8aa-TadA-8e-16aa-T7RNAP, ugi (pHW002)
247-X8aa-F	CTCTGGTAGCGGCTCTaacacgattaacatcgctaagaacg	Cloning of pBAD33-PmCDA1-16aa-TadA-8e-8aa-T7RNAP, ugi (pHW001)
248-X8aa-R	CCGGAACCATTAATGCTGCTCTGTGCTTTCTTTTGTG	Cloning of pBAD33-PmCDA1-16aa-TadA-8e-8aa-T7RNAP, ugi (pHW001)
254_pBAD-NdeI_rv	aatCATATGCCATGGtgaattcctc	Clonging of pBAD33-TadA-7.10-T7RNAP
255-TadA-A113-R	GCACCGCGCTTGCTATTAC	
314-F-seq-F2	CATTAGGAAGCAGCCCAGTAGTAG	sequencing primer for target gene
315-F-seq-R2	gagacgaaagggcccgtacg	sequencing primer for target gene
316-UGI-RBS-F	CAATAAATAAGGAGGATTTTTTatgaccaacctttccgacatcataggag	Cloning of pBAD33-PmCDA1-T7RNAP, (RBS) ugi (pDae069)
318-UGI-RBS-R	CTAGTACTCAAACAGAGCGCGCTCTGTTaggatccccgggctgcaG	Cloning of pBAD33-PmCDA1-T7RNAP, (RBS) ugi (pDae069)
341_NdeI_tadA_fw	TTAcatatgTTGTCTGAAGTCGAATTTAGCCAC	Clonging of pBAD33-TadA-7.10-T7RNAP
367_T7RNAP_taa_PstI_rv	ttatctgcagttacgcgaacgcgaagtcc	Clonging of pBAD33-TadA-7.10-T7RNAP
368_28b_th_rv	CATATGGCTGCCGCG	Clonging of pET28b-TadA-7.10-T7RNAP
369_28b_ov_tadA_fw	GGTGCCGCGCGGCAGCCATATGTTGTCTGAAGTCGAATTTAGCCACG	Clonging of pET28b-TadA-7.10-T7RNAP
370_tadA_XTEN_ov_rv	gtggcggactctgaggtcccgggagtctcgctgccgctATCCGTCGAGGATTGCG	Clonging of pET28b-TadA-7.10-T7RNAP
371_ov_XTEN_T7RNAP_fw	gactcccgggacctcagagtccgccacacccgaaagtaacacgattaacatcgctaagaac	Clonging of pET28b-TadA-7.10-T7RNAP

473_pBAD_dugi_fw__w187	aagcttggctgttttggc	Clonging of pBAD33-PmCDA1-T7RNAP (pDae014)

REFERENCES

Gaudelli NM, Komor AC, Rees HA, Packer MS, Badran AH, Bryson DI, Liu DR (2017) Programmable base editing of A*T to G*C in genomic DNA without DNA cleavage. *Nature* 551: 464-471

Melancon CE, 3rd, Schultz PG (2009) One plasmid selection system for the rapid evolution of aminoacyl-tRNA synthetases. *Bioorg Med Chem Lett* 19: 3845-3847

Park H, Kim S (2021) Gene-specific mutagenesis enables rapid continuous evolution of enzymes in vivo. *Nucleic Acids Res* 49: e32

Richter MF, Zhao KT, Eton E, Lapinaite A, Newby GA, Thuronyi BW, Wilson C, Koblan LW, Zeng J, Bauer DE *et al* (2020) Phage-assisted evolution of an adenine base editor with improved Cas domain compatibility and activity. *Nat Biotechnol* 38: 883-891