

Supplementary Information

Enhancement of Protein Thermostability by Three Consecutive Mutations Using Loop-Walking Method and Machine Learning

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Table S1. Random mutation primers for LPS-L1 to LPS-L12.

no.	mutation point	primer	sequence (5' - 3')
1	LPS-L1_A74/A75/T76	forward	<u>NNKNNKNNK</u> GGCGCAACCAAAGTTAACCTGGTTG
		reverse	CAGCACAGTTTTCACATACGCCAGC
2	LPS-L2_V199/G200/G201	forward	<u>NNKNNKNNK</u> AACACTCACCTGCTGTACTCTTGGGC
		reverse	AGTCTCGGTCGGCGCACC
3	LPS-L3_L127/A128/Y129	forward	<u>NNKNNKNNK</u> GACCCGACTGGCCTGTCTTCTACC
		reverse	AACGCCCTGAACGAAATCCGCG
4	LPS-L4_P216/T217/I218	forward	<u>NNKNNKNNK</u> TCTGTTTCGGTGTTACTGGTGCGAC
		reverse	CTGGATCGCAGTACCCGCCAAG
5	LPS-L5_S219/V220/F221	forward	<u>NNKNNKNNK</u> GGTGTTACTGGTGCGACTGATACCTCTAC
		reverse	GATGGTCGGCTGGATCGCAGTAC
6	LPS-L6_G222/V223/T224	forward	<u>NNKNNKNNK</u> GGTGCGACTGATACCTCTACTATCCCGC
		reverse	GAAAACAGAGATGGTCGGCTGGATCGC
7	LPS-L7_P233/L234/V235	forward	<u>NNKNNKNNK</u> GATCCGGCAAACGCACTGGACC
		reverse	GATAGTAGAGGTATCAGTCGCACCAGTAAC
8	LPS-L8_R258/G259/S260	forward	<u>NNKNNKNNK</u> GGTCAGAACGATGGTGTGGTGTCTAAGTG
		reverse	GTTCAACATAACGGTGCCGGTACCAAAC
9	LPS-L9_Q292/L293/L294	forward	<u>NNKNNKNNK</u> GGTGTTCTGGTGCTAACCGGAAGATC
		reverse	GTTGATCTCGTCCAGGTGGTTCCATTTGTAAGAG
10	LPS-L10_G25/V26/L27	forward	<u>NNKNNKNNK</u> GAGTACTGGTACGGTATTCAGGAAGACCTGC
		reverse	AGCATACTTATCGGTGCCAGTCAGACCATG
11	LPS-L11_P58/N59/G60	forward	<u>NNKNNKNNK</u> CGCGGCGAACAGCTGCTGGCGTATGTGAAAAC
		reverse	GCCGTCGTCGGAAGTGGAAACCAGACAGGTTT
12	LPS-L12_Q39/R40/G41	forward	<u>NNKNNKNNK</u> GCGACTGTTTACGTTGCGAACCTGTCTGGTTTC
		reverse	CTGCAGGTCTTCTGAATACCGTACCAGTACTC

Table S2. Mutation primers for LPS_Gly25.

mutation point	amino acid substitution	primer name	sequence (5' - 3')
Gly25	Ala	LPS_G25A-F1	<u>GCG</u> GTTCTGGAGTACTGGTACGGTATTCAGGAAGACC
	Cys	LPS_G25C-F1	<u>TGC</u> GTTCTGGAGTACTGGTACGGTATTCAGGAAGACC
	Asp	LPS_G25D-F1	<u>GAT</u> GTTCTGGAGTACTGGTACGGTATTCAGGAAGACC
	Glu	LPS_G25E-F1	<u>GAA</u> GTTCTGGAGTACTGGTACGGTATTCAGGAAGACC
	Phe	LPS_G25F-F1	<u>TTT</u> GTTCTGGAGTACTGGTACGGTATTCAGGAAGACC
	Gly	LPS_G25G-F1	<u>GGC</u> GTTCTGGAGTACTGGTACGGTATTCAGGAAGACC
	His	LPS_G25H-F1	<u>CAT</u> GTTCTGGAGTACTGGTACGGTATTCAGGAAGACC
	Ile	LPS_G25I-F1	<u>ATT</u> GTTCTGGAGTACTGGTACGGTATTCAGGAAGACC
	Lys	LPS_G25K-F1	<u>AAA</u> GTTCTGGAGTACTGGTACGGTATTCAGGAAGACC
	Leu	LPS_G25L-F1	<u>CTG</u> GTTCTGGAGTACTGGTACGGTATTCAGGAAGACC
	Met	LPS_G25M-F1	<u>ATG</u> GTTCTGGAGTACTGGTACGGTATTCAGGAAGACC
	Asn	LPS_G25N-F1	<u>AAC</u> GTTCTGGAGTACTGGTACGGTATTCAGGAAGACC
	Pro	LPS_G25P-F1	<u>CCG</u> GTTCTGGAGTACTGGTACGGTATTCAGGAAGACC
	Gln	LPS_G25Q-F1	<u>CAG</u> GTTCTGGAGTACTGGTACGGTATTCAGGAAGACC
	Arg	LPS_G25R-F1	<u>CGT</u> GTTCTGGAGTACTGGTACGGTATTCAGGAAGACC
	Ser	LPS_G25S-F1	<u>AGC</u> GTTCTGGAGTACTGGTACGGTATTCAGGAAGACC
	Thr	LPS_G25T-F1	<u>ACC</u> GTTCTGGAGTACTGGTACGGTATTCAGGAAGACC
	Val	LPS_G25V-F1	<u>GTG</u> GTTCTGGAGTACTGGTACGGTATTCAGGAAGACC
	Trp	LPS_G25W-F1	<u>TGG</u> GTTCTGGAGTACTGGTACGGTATTCAGGAAGACC
	Tyr	LPS_G25Y-F1	<u>TAT</u> GTTCTGGAGTACTGGTACGGTATTCAGGAAGACC
	-	reverse LPS_G25X-R1	AGCATACTTATCGGTGCCAGTCAGACCATGC

Table S3. Mutation primers for LPS_Val26.

mutation point	amino acid substitution	primer name	sequence (5' - 3')
Val26	Ala	LPS_V26A-F1	<u>GCG</u> CTGGAGTACTGGTACGGTATTCAGGAAGACCTG
	Cys	LPS_V26C-F1	<u>TGC</u> CTGGAGTACTGGTACGGTATTCAGGAAGACCTG
	Asp	LPS_V26D-F1	<u>GAT</u> CTGGAGTACTGGTACGGTATTCAGGAAGACCTG
	Glu	LPS_V26E-F1	<u>GAA</u> CTGGAGTACTGGTACGGTATTCAGGAAGACCTG
	Phe	LPS_V26F-F1	<u>TTC</u> CTGGAGTACTGGTACGGTATTCAGGAAGACCTG
	Gly	LPS_V26G-F1	<u>GGC</u> CTGGAGTACTGGTACGGTATTCAGGAAGACCTG
	His	LPS_V26H-F1	<u>CAT</u> CTGGAGTACTGGTACGGTATTCAGGAAGACCTG
	Ile	LPS_V26I-F1	<u>ATT</u> CTGGAGTACTGGTACGGTATTCAGGAAGACCTG
	Lys	LPS_V26K-F1	<u>AAA</u> CTGGAGTACTGGTACGGTATTCAGGAAGACCTG
	Leu	LPS_V26L-F1	<u>CTG</u> CTGGAGTACTGGTACGGTATTCAGGAAGACCTG
	Met	LPS_V26M-F1	<u>ATG</u> CTGGAGTACTGGTACGGTATTCAGGAAGACCTG
	Asn	LPS_V26N-F1	<u>AAC</u> CTGGAGTACTGGTACGGTATTCAGGAAGACCTG
	Pro	LPS_V26P-F1	<u>CCG</u> CTGGAGTACTGGTACGGTATTCAGGAAGACCTG
	Gln	LPS_V26Q-F1	<u>CAG</u> CTGGAGTACTGGTACGGTATTCAGGAAGACCTG
	Arg	LPS_V26R-F1	<u>CGT</u> CTGGAGTACTGGTACGGTATTCAGGAAGACCTG
	Ser	LPS_V26S-F1	<u>AGC</u> CTGGAGTACTGGTACGGTATTCAGGAAGACCTG
	Thr	LPS_V26T-F1	<u>ACC</u> CTGGAGTACTGGTACGGTATTCAGGAAGACCTG
	Val	LPS_V26V-F1	<u>GTG</u> CTGGAGTACTGGTACGGTATTCAGGAAGACCTG
	Trp	LPS_V26W-F1	<u>TGG</u> CTGGAGTACTGGTACGGTATTCAGGAAGACCTG
	Tyr	LPS_V26Y-F1	<u>TAT</u> CTGGAGTACTGGTACGGTATTCAGGAAGACCTG
	-	reverse LPS_V26X-R1	ACCAGCATACTTATCGGTGCCAGTCAGACC

Table S4. Mutation primers for LPS_Leu27.

mutation point	amino acid substitution	primer name	sequence (5' - 3')
Leu27	Ala	LPS_L27A-F1	<u>GCG</u> GAGTACTGGTACGGTATTCAGGAAGACCTGCAG
	Cys	LPS_L27C-F1	<u>TGC</u> GAGTACTGGTACGGTATTCAGGAAGACCTGCAG
	Asp	LPS_L27D-F1	<u>GAT</u> GAGTACTGGTACGGTATTCAGGAAGACCTGCAG
	Glu	LPS_L27E-F1	<u>GAA</u> GAGTACTGGTACGGTATTCAGGAAGACCTGCAG
	Phe	LPS_L27F-F1	<u>TTC</u> GAGTACTGGTACGGTATTCAGGAAGACCTGCAG
	Gly	LPS_L27G-F1	<u>GGC</u> GAGTACTGGTACGGTATTCAGGAAGACCTGCAG
	His	LPS_L27H-F1	<u>CAT</u> GAGTACTGGTACGGTATTCAGGAAGACCTGCAG
	Ile	LPS_L27I-F1	<u>ATT</u> GAGTACTGGTACGGTATTCAGGAAGACCTGCAG
	Lys	LPS_L27K-F1	<u>AAA</u> GAGTACTGGTACGGTATTCAGGAAGACCTGCAG
	Leu	LPS_L27L-F1	<u>CTG</u> GAGTACTGGTACGGTATTCAGGAAGACCTGCAG
	Met	LPS_L27M-F1	<u>ATG</u> GAGTACTGGTACGGTATTCAGGAAGACCTGCAG
	Asn	LPS_L27N-F1	<u>AAC</u> GAGTACTGGTACGGTATTCAGGAAGACCTGCAG
	Pro	LPS_L27P-F1	<u>CCG</u> GAGTACTGGTACGGTATTCAGGAAGACCTGCAG
	Gln	LPS_L27Q-F1	<u>CAG</u> GAGTACTGGTACGGTATTCAGGAAGACCTGCAG
	Arg	LPS_L27R-F1	<u>CGT</u> GAGTACTGGTACGGTATTCAGGAAGACCTGCAG
	Ser	LPS_L27S-F1	<u>AGC</u> GAGTACTGGTACGGTATTCAGGAAGACCTGCAG
	Thr	LPS_L27T-F1	<u>ACC</u> GAGTACTGGTACGGTATTCAGGAAGACCTGCAG
	Val	LPS_L27V-F1	<u>GTG</u> GAGTACTGGTACGGTATTCAGGAAGACCTGCAG
	Trp	LPS_L27W-F1	<u>TGG</u> GAGTACTGGTACGGTATTCAGGAAGACCTGCAG
	Tyr	LPS_L27Y-F1	<u>TAT</u> GAGTACTGGTACGGTATTCAGGAAGACCTGCAG
	-	reverse LPS_L27X-R1	AACACCAGCATACTTATCGGTGCCAGTCAGAC

Table S5. Mutation primers for LPS_Pro233.

mutation point	amino acid substitution	primer name	sequence (5' - 3')
Pro233	Ala	LPS_P233A-F1	<u>GCG</u> CTGGTGGATCCGGCAAACGCACTG
	Cys	LPS_P233C-F1	<u>TGC</u> CTGGTGGATCCGGCAAACGCACTG
	Asp	LPS_P233D-F1	<u>GAT</u> CTGGTGGATCCGGCAAACGCACTG
	Glu	LPS_P233E-F1	<u>GAA</u> CTGGTGGATCCGGCAAACGCACTG
	Phe	LPS_P233F-F1	<u>TTC</u> CTGGTGGATCCGGCAAACGCACTG
	Gly	LPS_P233G-F1	<u>GGC</u> CTGGTGGATCCGGCAAACGCACTG
	His	LPS_P233H-F1	<u>CAT</u> CTGGTGGATCCGGCAAACGCACTG
	Ile	LPS_P233I-F1	<u>ATT</u> CTGGTGGATCCGGCAAACGCACTG
	Lys	LPS_P233K-F1	<u>AAA</u> CTGGTGGATCCGGCAAACGCACTG
	Leu	LPS_P233L-F1	<u>CTG</u> CTGGTGGATCCGGCAAACGCACTG
	Met	LPS_P233M-F1	<u>ATG</u> CTGGTGGATCCGGCAAACGCACTG
	Asn	LPS_P233N-F1	<u>AAC</u> CTGGTGGATCCGGCAAACGCACTG
	Pro	LPS_P233P-F1	<u>CCG</u> CTGGTGGATCCGGCAAACGCACTG
	Gln	LPS_P233Q-F1	<u>CAG</u> CTGGTGGATCCGGCAAACGCACTG
	Arg	LPS_P233R-F1	<u>CGT</u> CTGGTGGATCCGGCAAACGCACTG
	Ser	LPS_P233S-F1	<u>AGC</u> CTGGTGGATCCGGCAAACGCACTG
	Thr	LPS_P233T-F1	<u>ACC</u> CTGGTGGATCCGGCAAACGCACTG
	Val	LPS_P233V-F1	<u>GTG</u> CTGGTGGATCCGGCAAACGCACTG
	Trp	LPS_P233W-F1	<u>TGG</u> CTGGTGGATCCGGCAAACGCACTG
	Tyr	LPS_P233Y-F1	<u>TAT</u> CTGGTGGATCCGGCAAACGCACTG
	-	reverse LPS_P233X-R1	GATAGTAGAGGTATCAGTCGCACCAGTAACACCG

Table S6. Mutation primers for LPS_Leu234.

mutation point	amino acid substitution	primer name	sequence (5' - 3')
Leu234	Ala	LPS_L234A-F1	<u>GCG</u> GTGGATCCGGCAAACGCACTGGAC
	Cys	LPS_L234C-F1	<u>TGC</u> GTGGATCCGGCAAACGCACTGGAC
	Asp	LPS_L234D-F1	<u>GAT</u> GTGGATCCGGCAAACGCACTGGAC
	Glu	LPS_L234E-F1	<u>GAA</u> GTGGATCCGGCAAACGCACTGGAC
	Phe	LPS_L234F-F1	<u>TTC</u> GTGGATCCGGCAAACGCACTGGAC
	Gly	LPS_L234G-F1	<u>GGC</u> GTGGATCCGGCAAACGCACTGGAC
	His	LPS_L234H-F1	<u>CAT</u> GTGGATCCGGCAAACGCACTGGAC
	Ile	LPS_L234I-F1	<u>ATT</u> GTGGATCCGGCAAACGCACTGGAC
	Lys	LPS_L234K-F1	<u>AAA</u> GTGGATCCGGCAAACGCACTGGAC
	Leu	LPS_L234L-F1	<u>CTG</u> GTGGATCCGGCAAACGCACTGGAC
	Met	LPS_L234M-F1	<u>ATG</u> GTGGATCCGGCAAACGCACTGGAC
	Asn	LPS_L234N-F1	<u>AAC</u> GTGGATCCGGCAAACGCACTGGAC
	Pro	LPS_L234P-F1	<u>CCG</u> GTGGATCCGGCAAACGCACTGGAC
	Gln	LPS_L234Q-F1	<u>CAG</u> GTGGATCCGGCAAACGCACTGGAC
	Arg	LPS_L234R-F1	<u>CGT</u> GTGGATCCGGCAAACGCACTGGAC
	Ser	LPS_L234S-F1	<u>AGC</u> GTGGATCCGGCAAACGCACTGGAC
	Thr	LPS_L234T-F1	<u>ACC</u> GTGGATCCGGCAAACGCACTGGAC
	Val	LPS_L234V-F1	<u>GTG</u> GTGGATCCGGCAAACGCACTGGAC
	Trp	LPS_L234W-F1	<u>TGG</u> GTGGATCCGGCAAACGCACTGGAC
	Tyr	LPS_L234Y-F1	<u>TAT</u> GTGGATCCGGCAAACGCACTGGAC
	-	reverse LPS_L234X-R1	CGGGATAGTAGAGGTATCAGTCGCACCAGTAAC

Table S7. Mutation primers for LPS_Val235.

mutation point	amino acid substitution	primer name	sequence (5' - 3')
Val235	Ala	LPS_V235A-F1	<u>G</u> CGGATCCGGCAAACGCACTGGACCC
	Cys	LPS_V235C-F1	<u>T</u> GCGATCCGGCAAACGCACTGGACCC
	Asp	LPS_V235D-F1	<u>G</u> ATGATCCGGCAAACGCACTGGACCC
	Glu	LPS_V235E-F1	<u>G</u> AAGATCCGGCAAACGCACTGGACCC
	Phe	LPS_V235F-F1	<u>T</u> TCGATCCGGCAAACGCACTGGACCC
	Gly	LPS_V235G-F1	<u>G</u> GCGATCCGGCAAACGCACTGGACCC
	His	LPS_V235H-F1	<u>C</u> ATGATCCGGCAAACGCACTGGACCC
	Ile	LPS_V235I-F1	<u>A</u> TTGATCCGGCAAACGCACTGGACCC
	Lys	LPS_V235K-F1	<u>A</u> AAAGATCCGGCAAACGCACTGGACCC
	Leu	LPS_V235L-F1	<u>C</u> TGGATCCGGCAAACGCACTGGACCC
	Met	LPS_V235M-F1	<u>A</u> TGGATCCGGCAAACGCACTGGACCC
	Asn	LPS_V235N-F1	<u>A</u> ACGATCCGGCAAACGCACTGGACCC
	Pro	LPS_V235P-F1	<u>C</u> CGATCCGGCAAACGCACTGGACCC
	Gln	LPS_V235Q-F1	<u>C</u> AGGATCCGGCAAACGCACTGGACCC
	Arg	LPS_V235R-F1	<u>C</u> GTGATCCGGCAAACGCACTGGACCC
	Ser	LPS_V235S-F1	<u>A</u> GCGATCCGGCAAACGCACTGGACCC
	Thr	LPS_V235T-F1	<u>A</u> CCGATCCGGCAAACGCACTGGACCC
	Val	LPS_V235V-F1	<u>G</u> TGGATCCGGCAAACGCACTGGACCC
	Trp	LPS_V235W-F1	<u>T</u> GGGATCCGGCAAACGCACTGGACCC
	Tyr	LPS_V235Y-F1	<u>T</u> ATGATCCGGCAAACGCACTGGACCC
	-	reverse	LPS_V235X-R1 CAGCGGGATAGTAGAGGTATCAGTCGCACC

Table S8. Top 20 ranking predicted to fit the rule of "high improvement".^a

ranking	P233X/L234X/V235X	categories of prediction	
		medium	high
1	DPE	0.027	0.973
2	EPE	0.027	0.973
3	DPG	0.030	0.970
4	EPG	0.031	0.969
5	DPD	0.031	0.969
6	DPN	0.031	0.969
7	EPD	0.032	0.968
8	EPN	0.032	0.968
9	DGE	0.036	0.964
10	EGE	0.036	0.964
11	DPS	0.040	0.960
12	DGG	0.040	0.960
13	EPS	0.040	0.960
14	DNE	0.040	0.960
15	EGG	0.041	0.959
16	ENE	0.041	0.959
17	DKE	0.041	0.959
18	DGD	0.041	0.959
19	DGN	0.042	0.958
20	DQE	0.042	0.958

^a The numbers in categories of prediction designate probabilities for category prediction.

Table S9. 20 ranking predicted to fit the rule of "medium improvement".^a

ranking	P233X/L234X/V235X	categories of prediction	
		medium	high
2637	MWG	0.445	0.555
2638	HQA	0.445	0.555
2639	ADT	0.445	0.555
2640	ISN	0.445	0.555
2641	MNA	0.445	0.555
2642	HFN	0.445	0.555
2643	VCE	0.446	0.554
2644	HKK	0.446	0.554
2645	QYM	0.446	0.554
2646	HWN	0.446	0.554
2647	HES	0.446	0.554
2648	AAR	0.446	0.554
2649	NEL	0.447	0.553
2650	HMD	0.447	0.553
2651	MMG	0.447	0.553
2652	QVM	0.447	0.553
2653	AET	0.448	0.552
2654	NSY	0.448	0.552
2655	KNE	0.449	0.551
2656	HQK	0.449	0.551

^a The numbers in categories of prediction designate probabilities for category prediction.

Table S10. Mutation primers for high 20 mutants.

prediction ranking	strain	primer name	sequence (5' - 3')
1	LPS_P233D/L234P/V235E	LPS_DPE-F1	<u>GATCCGGAA</u> GATCCGGCAAACGCACTGGACC
2	LPS_P233E/L234P/V235E	LPS_EPE-F1	<u>GAACCGGAA</u> GATCCGGCAAACGCACTGGACC
3	LPS_P233D/L234P/V235G	LPS_DPG-F1	<u>GATCCGGGCG</u> GATCCGGCAAACGCACTGGACC
4	LPS_P233E/L234P/V235G	LPS_EPG-F1	<u>GAACCGGGCG</u> GATCCGGCAAACGCACTGGACC
5	LPS_P233D/L234P/V235D	LPS_DPD-F1	<u>GATCCGGAT</u> GATCCGGCAAACGCACTGGACC
6	LPS_P233D/L234P/V235N	LPS_DPN-F1	<u>GATCCGAAC</u> GATCCGGCAAACGCACTGGACC
7	LPS_P233E/L234P/V235D	LPS_EPD-F1	<u>GAACCGGAT</u> GATCCGGCAAACGCACTGGACC
8	LPS_P233E/L234P/V235N	LPS_EPN-F1	<u>GAACCGAAC</u> GATCCGGCAAACGCACTGGACC
9	LPS_P233D/L234G/V235E	LPS_DGE-F1	<u>GATGGCGAA</u> GATCCGGCAAACGCACTGGACC
10	LPS_P233E/L234G/V235E	LPS_EGE-F1	<u>GAAGGCGAA</u> GATCCGGCAAACGCACTGGACC
11	LPS_P233D/L234P/V235S	LPS_DPS-F1	<u>GATCCGAGCG</u> GATCCGGCAAACGCACTGGACC
12	LPS_P233D/L234G/V235G	LPS_DGG-F1	<u>GATGGCGGCG</u> GATCCGGCAAACGCACTGGACC
13	LPS_P233E/L234P/V235S	LPS_EPS-F1	<u>GAACCGAGCG</u> GATCCGGCAAACGCACTGGACC
14	LPS_P233D/L234N/V235E	LPS_DNE-F1	<u>GATAACGAA</u> GATCCGGCAAACGCACTGGACC
15	LPS_P233E/L234G/V235G	LPS_EGG-F1	<u>GAAGGCGGCG</u> GATCCGGCAAACGCACTGGACC
16	LPS_P233E/L234N/V235E	LPS_ENE-F1	<u>GA AAAACGAA</u> GATCCGGCAAACGCACTGGACC
17	LPS_P233D/L234K/V235E	LPS_DKE-F1	<u>GATAAAGAA</u> GATCCGGCAAACGCACTGGACC
18	LPS_P233D/L234G/V235D	LPS_DGD-F1	<u>GATGGCGAT</u> GATCCGGCAAACGCACTGGACC
19	LPS_P233D/L234G/V235N	LPS_DGN-F1	<u>GATGGCAAC</u> GATCCGGCAAACGCACTGGACC
20	LPS_P233D/L234Q/V235E	LPS_DQE-F1	<u>GATCAGGAA</u> GATCCGGCAAACGCACTGGACC
-	-	reverse	LPS-L7-R1 GATAGTAGAGGTATCAGTCGCACCACTAAC

Table S11. Mutation primers for medium 20 mutants.

prediction ranking	strain	primer name	sequence (5' - 3')
2637	LPS_P233M/L234W/V235G	LPS_MWG-F1	<u>ATGTGGGGCG</u> GATCCGGCAAACGCACTGGACC
2638	LPS_P233H/L234Q/V235A	LPS_HQA-F1	<u>CATCAGGCG</u> GATCCGGCAAACGCACTGGACC
2639	LPS_P233A/L234D/V235T	LPS_ADT-F1	<u>GCGGATACCG</u> GATCCGGCAAACGCACTGGACC
2640	LPS_P233I/L234S/V235N	LPS_ISN-F1	<u>ATTAGCAAC</u> GATCCGGCAAACGCACTGGACC
2641	LPS_P233M/L234N/V235A	LPS_MNA-F1	<u>ATGAACGCG</u> GATCCGGCAAACGCACTGGACC
2642	LPS_P233H/L234F/V235N	LPS_HFN-F1	<u>CATTTCAAC</u> GATCCGGCAAACGCACTGGACC
2643	LPS_P233V/L234C/V235E	LPS_VCE-F1	<u>GTGTGCGAA</u> GATCCGGCAAACGCACTGGACC
2644	LPS_P233H/L234K/V235K	LPS_HKK-F1	<u>CATAAAAAA</u> GATCCGGCAAACGCACTGGACC
2645	LPS_P233Q/L234Y/V235M	LPS_QYM-F1	<u>CAGTATATG</u> GATCCGGCAAACGCACTGGACC
2646	LPS_P233H/L234W/V235N	LPS_HWN-F1	<u>CATTGGAAC</u> GATCCGGCAAACGCACTGGACC
2647	LPS_P233H/L234E/V235S	LPS_HES-F1	<u>CATGAAAGC</u> GATCCGGCAAACGCACTGGACC
2648	LPS_P233A/L234A/V235R	LPS_AAR-F1	<u>GCGGCGCGT</u> GATCCGGCAAACGCACTGGACC
2649	LPS_P233N/L234E/V235L	LPS_NEL-F1	<u>AACGAACTG</u> GATCCGGCAAACGCACTGGACC
2650	LPS_P233H/L234M/V235D	LPS_HMD-F1	<u>CATATGGAT</u> GATCCGGCAAACGCACTGGACC
2651	LPS_P233M/L234M/V235G	LPS_MMG-F1	<u>ATGATGGGCG</u> GATCCGGCAAACGCACTGGACC
2652	LPS_P233Q/L234V/V235M	LPS_QVM-F1	<u>CAGGTGATG</u> GATCCGGCAAACGCACTGGACC
2653	LPS_P233A/L234E/V235T	LPS_AET-F1	<u>GCGGAAACC</u> GATCCGGCAAACGCACTGGACC
2654	LPS_P233N/L234S/V235Y	LPS_NSY-F1	<u>AACAGCTAT</u> GATCCGGCAAACGCACTGGACC
2655	LPS_P233K/L234N/V235E	LPS_KNE-F1	<u>AAAAACGAA</u> GATCCGGCAAACGCACTGGACC
2656	LPS_P233H/L234Q/V235K	LPS_HQK-F1	<u>CATCAGAAA</u> GATCCGGCAAACGCACTGGACC
-	-	reverse	LPS-L7-R1 GATAGTAGAGGTATCAGTCGCACCACTAAC

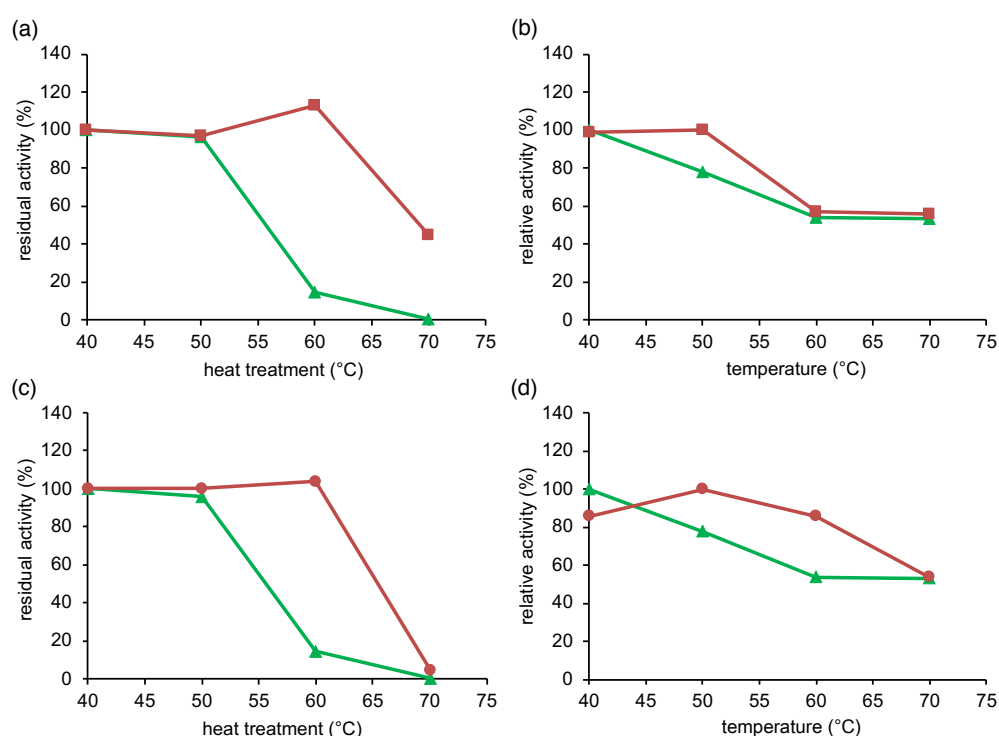


Figure S1. (a) Thermostability and (b) optimum temperature of the P233G/L234E/V235M mutant and (c) thermostability and (d) optimum temperature of the P233H/L234V/V235H mutant. In (a) and (c), horizontal axis: temperature of heat treatment for 30 min, vertical axis: relative activity after heat treatment. In (b) and (d), horizontal axis: assay temperature, vertical axis: relative enzymatic activity. ▲: LPS_wild-type, ■: P233G/L234E/V235M, ●: P233H/L234V/V235H.

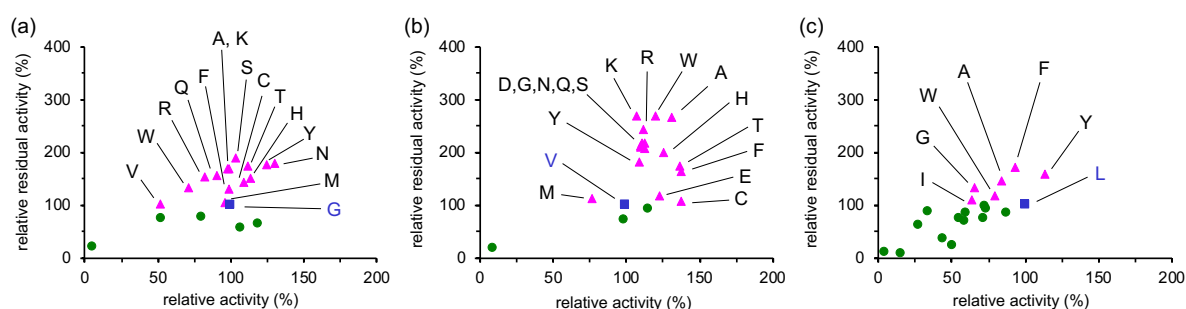


Figure S2. Thermostability plots for (a) G25X, (b) V26X, and (c) L27X single mutants using relative activity without heat treatment (horizontal axis) and relative residual activity after heat treatment at 60 °C for 30 min (vertical axis). The blue square represents the wild-type enzyme while the pink triangle represents the mutants with improved thermostability, and the green circle represents the mutants with reduced thermostability.

AAindex No.	name	A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
401	Isoelectric point (Zimmerman et al., 1968)	6	5	3	3	5	2	8	6	10	6	6	5	6	6	11	6	6	6	6	6
80	Normalized van der Waals volume (Fauchere et al., 1988)	1	2	3	4	6	2	5	4	5	4	4	3	3	4	6	2	3	3	8	6
99	Alpha-helix indices for beta-proteins (Geisow-Roberts, 1980)	2	1	2	2	0	2	1	1	1	1	1	1	0	1	0	1	1	1	2	1
102	Beta-strand indices for beta-proteins (Geisow-Roberts, 1980)	1	1	1	0	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1
444	Side-chain contribution to protein stability (kJ/mol) (Takano-Yutani, 2001)	10	3	5	4	23	2	12	17	11	17	12	4	15	2	7	3	7	15	24	17
510	The stability scale from the knowledge-based atom-atom potential (Zhou-Zhou, 2004)	2	4	2	2	6	2	3	5	2	5	4	2	2	2	3	2	2	4	6	5
151	Hydropathy index (Kyte-Doolittle, 1982)	2	3	4	4	3	2	3	5	4	4	2	4	2	4	5	1	1	4	1	1
62	Normalized frequency of turn (Crawford et al., 1973)	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	0	1	1
432	Free energy in beta-strand region (Munoz-Serrano, 1994)	1	1	1	1	1	2	1	1	1	1	1	1	1	3	1	1	1	0	1	1
430	Free energy in alpha-helical region (Munoz-Serrano, 1994)	1	1	1	1	1	2	1	1	1	1	1	1	1	2	1	1	1	1	1	1
400	Polarity (Zimmerman et al., 1968)	0	1	50	50	0	2	52	0	50	0	1	3	2	4	52	2	2	0	2	2
148	Side chain interaction parameter (Krigbaum-Komoriya, 1979)	4	2	6	6	3	2	6	2	8	4	2	6	7	6	7	5	5	3	3	4
136	Amino acid distribution (Jukes et al., 1975)	5	1	4	3	2	2	1	3	4	5	1	3	3	2	3	5	4	4	1	2

Figure S3. AAindex.