Supporting Information

Table of Contents

I. Primers

1. For library construction.

pBK-TyrRS-Nde1-F1
TTGAGGAATCCCATATGGACGA

TyrRS-Lib1-R1
CAATTCCTTATTTTTAAATAA

TyrRS-Lib1-F2
TTATTTAAAAATAAGGAATTGNNKCCANNKNNKTTAAAAAATGCTGTAGCTGAAG

TyrRS-Lib1-R2
GCGAACGCCTTATCCGGCCTG

TyrRS-F261-R
TTTTTCTGGCCTTTTTATGGT

TyrRS-F261-F
ACCATAAAAAGGCCAGAAAAANNKGGTGGAGATTTGACAGTTAATA

pBK-wtAcF-D286R-F2
AAAAATAAGGAATTGCATCCAATGCGCTTAAAAAATGCTG

2. For mutation transfer

TTGAGGAATCCCATATGGACGA

CTCCATCCCTCCAACAGC

GCTGTTGGAGGGATGGAG

GCGAACGCCTTATCCGGCCTG

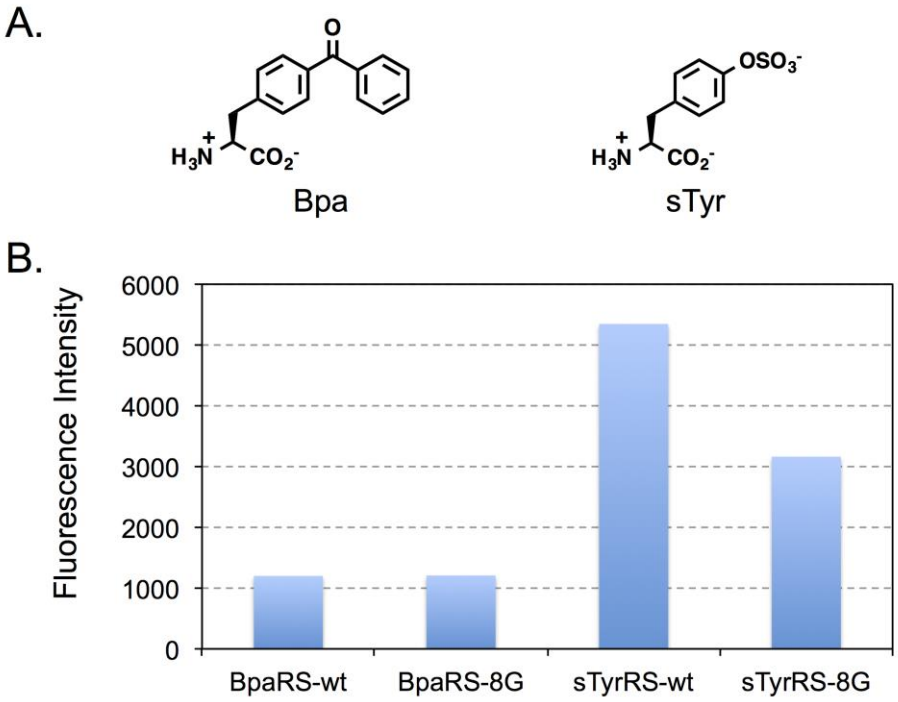

Figure S1. GFP fluorescence assays of cells expressing BpaRS and sTyrRS variants that contain anticodon recognition region mutations from AcPheRS-8G.

(A) The structures of *p*-benzoyl-L-phenylalanine (Bpa) and sulfotyrosine (sTyr); (B) Fluorescence readings of *E. coli* GeneHogs cells expressing BpaRS and sTyrRS variants, each co-expressed with $MjtRNA^{Tyr}_{CUA}$, in the presence of 1 mM Bpa and 10 mM sTyr, respectively. Fluorescence intensity was normalized to cell growth.

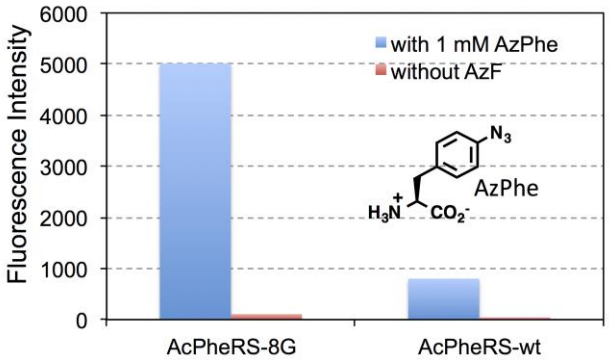

Figure S2. GFP fluorescence assays of AzPhe incorporation by AcPheRS-wt and AcPheRS-8G. Fluorescence readings of *E. coli* GeneHogs cells expressing AcPheRS-wt and AcPheRS-8G, each co-expressed with $MjtRNA^{Tyr}_{CUA}$, in the presence or the absence of 1 mM AzPhe. Fluorescence intensity was normalized to cell growth.

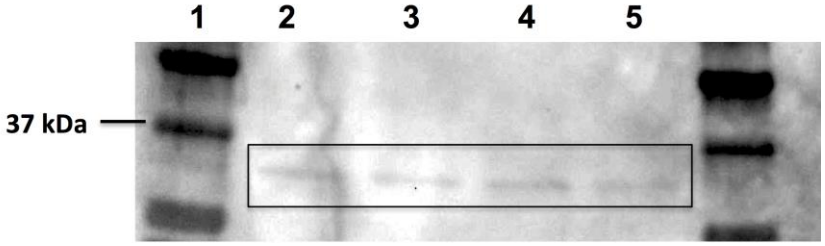

Figure S3. Western blot analysis of protein expression level. Lane 1, marker; lane 2, AcPheRS-8G; lane 3, AcPheRS-8G; lane 4, AcPheRS-wt; lane 5, AcPheRS-wt.

The AcPheRS-8G and AcPheRS-wt were sub-cloned and expressed as C-terminus His_6 fusion proteins. Each sample lane was normalized to the same number of cells based on OD_{600nm} measurement. Protein expression experiments were conducted as duplicates for each protein. The protein bands were visualized immunochemically using anti-His antibody and HRP-labeled secondary antibody. No significant differences in protein expression level were observed between AcPheRS-8G and AcPheRS-wt.

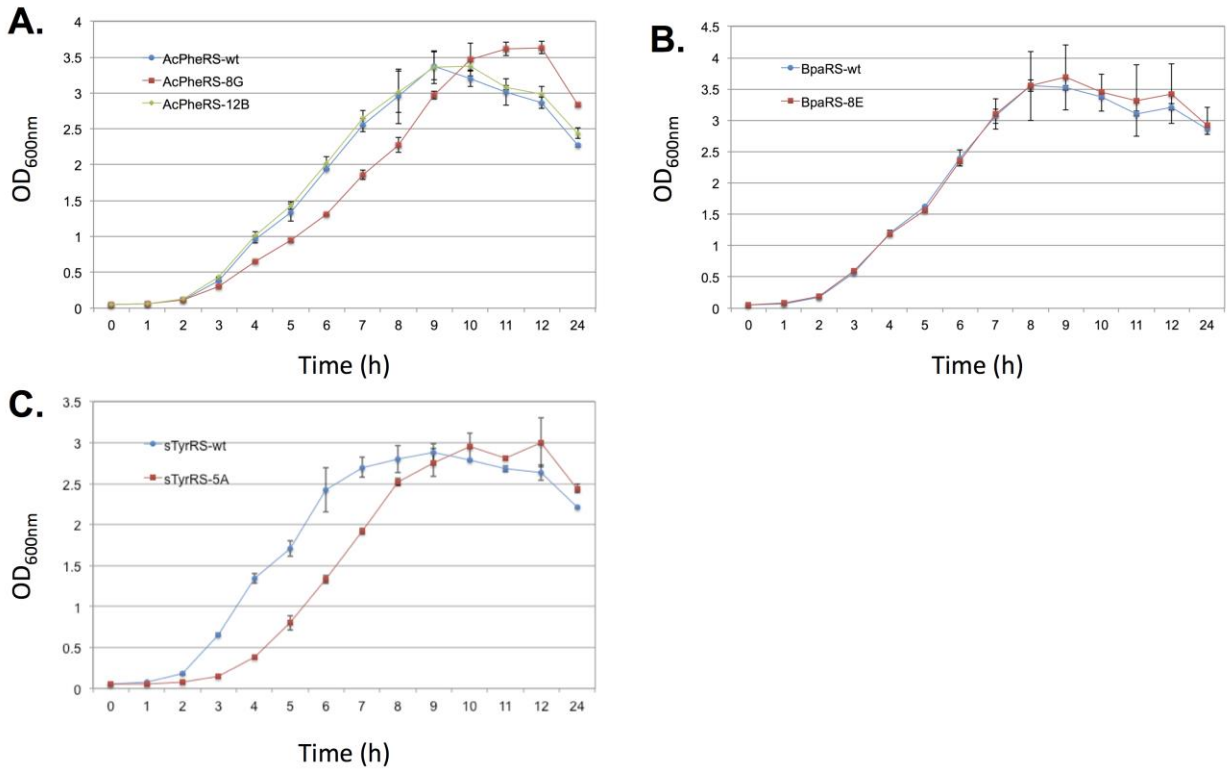

Figure S4. Growth rate of *E. coli* cells that contain different *Mj*TyrRS variants.

Data are the average of three experiments.

Note. Similar cell growth rates of strains harboring different AcPheRS variants were observed. It is therefore unlikely that the evolved aminoacyl-tRNA synthetase mutants mis-aminoacylate endogenous tRNAs at detectable higher level than their parent aminoacyl-tRNA synthetases. Otherwise, stronger cell growth inhibition would be observed in the presence of the evolved aminoacyl-tRNA synthetase mutants.