Supporting information

Materials and Methods

Isopropyl-β-D-thiogalactoside (IPTG) was purchased from Anatrace, L-arabinose was purchased from Biosynth, and nicotinamide was purchased from Sigma. PfuUltra II Fusion HS DNA Polymerase was obtained from Agilent Technologies; Gibson assembly master mix and dNTPs were obtained from New England Biolabs; oligonucleotide primers were purchased from Integrated DNA Technologies. Plasmid DNA preparation was carried out with the ZR Plasmid Miniprep-Classic Kit (Zymo research). Antibiotics were obtained from Corning-Mediatech. MG1655 strain was ordered from ATCC, ΔcobB strain was obtained from CGSC (#9039). A Fisher scientific 550 sonic disruptor was used. AcetylLysine was purchased from Alfa Aesar. ncAA stock solution (100 mM in water) was sterilized with a 0.22 μM filter. Optical density was measured with Ultrospec 10 Cell density meter. Absorbance and emission spectra were measured on plater reader Varioskan Lux.

1. Purification procedure for recombinant protein with genetically encoded AcK.

   E. coli MG1655 was co-transformed with pUltra-AcKRS-tRNA$_{CUA}$ (Spec^R)[1] and pET30b-T5-TEM1-K73TAG (Kan^R). A single colony was picked and inoculated into 5 mL LB, and grown overnight at 37 °C. The saturated culture was diluted 200-fold (125 μL to 25 mL) with LB and grown at 37 °C. When the OD reached 0.5, 5 mM AcK was added and the culture was grown for another 0.5 h before induction with 1 mM IPTG, and supplemented with or without 20 mM NAM. The induced cells were grown an additional 6 h at 37 °C. The cells were pelleted and lysed with a sonic disruptor in the presence of protease inhibitors, and insoluble protein and cell debris were removed by centrifugation. The protein was purified by Ni^{2+} affinity chromatography (according to the manufacture's protocol, QIAGEN), and finally buffer exchanged with an Amicon centrifugal filter.

2. Determination of the escape frequency.

   E. coli MG1655 was co-transformed with a pBK vector containing TEM1-K73TAG-barnase(2TAA) (or TEM1-K73TAG, TEM1-N52TAG) and a pUltra vector containing AcKRS/tRNA$_{CUA}$, and plated on LB agar plates supplemented with 50 μg/mL kanamycin and 50 μg/mL spectinomycin. Colonies were picked in triplicate and inoculated in LB medium. Overnight 37 °C cultures were diluted 1:1000 to start new 10 mL cultures in LB medium with 100 μg/mL ampicillin, 25 μg/mL kanamycin, 25 μg/mL spectinomycin, 4 mM AcK, 1 mM IPTG. The cultures were grown at 37 °C until saturation, then diluted 1:1000 to start another round of growth. After iterative growth for 100 generations, the cultures were grown in 100 mL LB medium to deplete intracellular AcK. 2 x 10^{10} cells were then plated on LB agar plates containing 100 μg/mL ampicillin and 25 μg/mL kanamycin to determine the escape frequency after 24 hours at 37 °C.

3. Evolving PylRS mutant for PrK, BuK and CrK with TEM1-K73TAG-based selection assay.

E. coli DH10B harboring pRep-PylT-TEM-K73TAG (Tet^R) was transformed with the PylRS mutant library in pBK vector, and plated on LB agar plate in the presence of 50 μg/mL kanamycin and 12.5 μg/mL tetracycline. Over 5×10^8 colonies were obtained and harvested, and 1×10^7 colonies (theoretical library size ~ 1×10^6) were plated on a LB agar plate containing 50 μg/mL kanamycin and 12.5 μg/mL tetracycline, 50 μg/mL ampicillin and 2 mM ncAA. The selection plate was incubated at 37 °C for 16 hours, and obtained single colonies were picked for sequencing.

Fig. S1Viability of *E. coli* MG1655 harboring TEM1-K234TAG and AcKRS/$tRNA_{CUA}$ on LB agar plates at 37 °C after 16 hours. The positive control contained the wild type TEM1 gene; 2mm AcK, 1 mM IPTG and 20 mm NAM were used respectively.

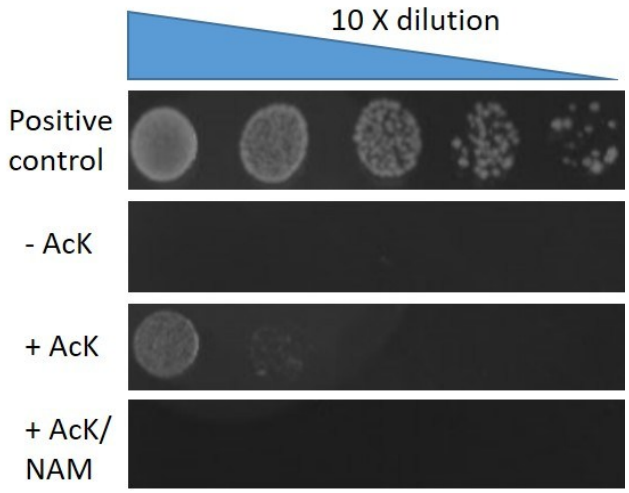

Fig. S2 Direct deacetylation of β-lactamase-K73AcK by cobB in the presence of NAD^+. The reaction was carried out with 2.5 µM β-lactamase-K73AcK, 29 nM cobB and 300 µM NAD^+ in 10 mM pH 7.4 phosphate buffer at 37 °C. Samples were analyzed by LC-ESI-QTOF mass spectrometry after incubation for 16 hours. CobB was expressed in *E. coli* with an N-terminal His-tag, and verified by mass spectrometry (Exp. 27539, Obs. 27539).

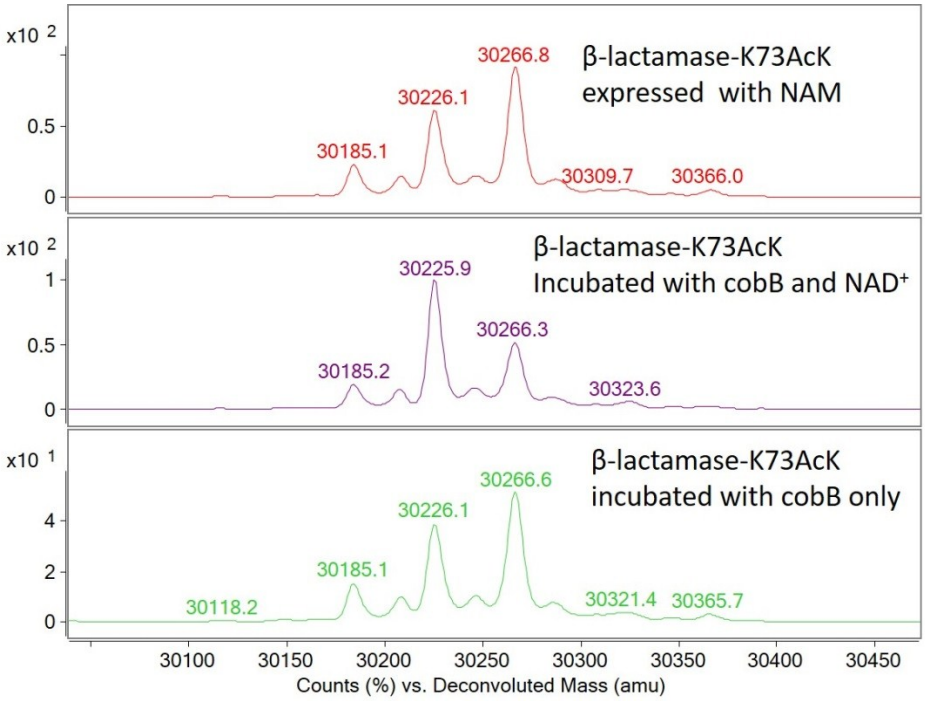

Fig. S3 K73 in β-lactamase in a pBK vector was mutated to the other 19 natural amino acids, and introduced into *E. coli* MG1655 strain. The cell culture after serial dilution was spotted on LB agar plate in the presence of 100 μg/mL ampicillin. The plate was kept at 37 ^{o}C for 16 hours.

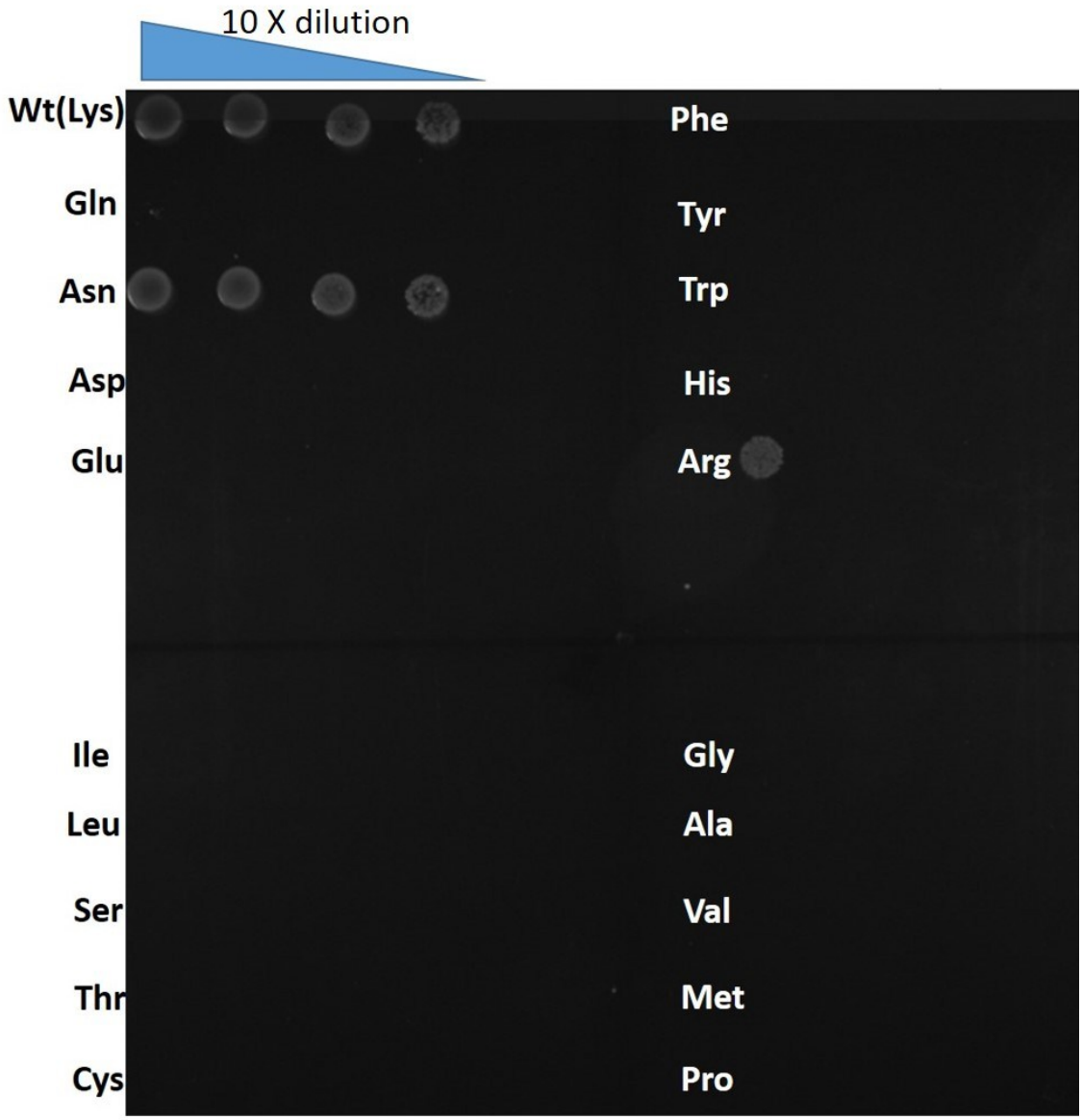

Fig. S4 Evaluation of the toxicity of the barnase-based conditional suicide switch to the escape strain. Wild type *E. coli* MG1655 strain and the escape strain were transformed with pNeg-Barnase-K27TAA-K62TAA. Equal amounts of cell culture were applied to LB agar plates containing 100 μg/mL ampicillin in the presence or absence of 0.2% arabinose, and kept at 37 °C for 16 hours. The barnase gene was under the control of an araBAD promoter.

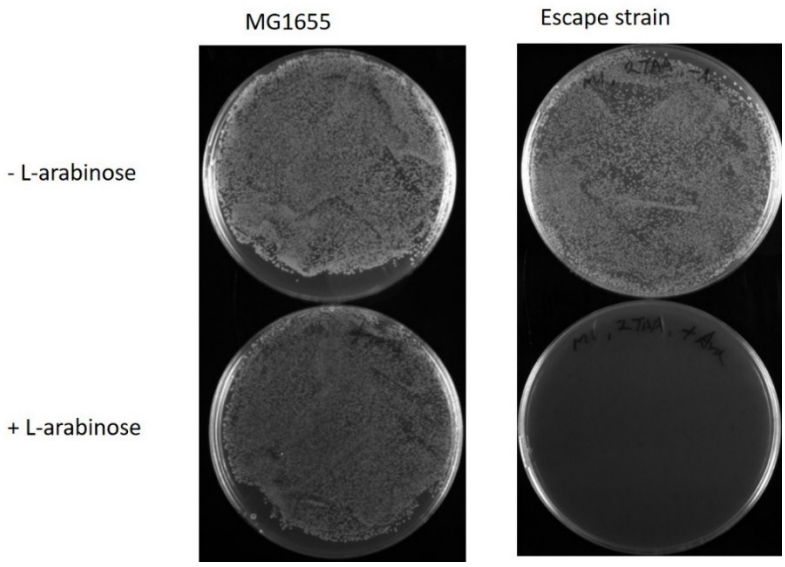

Fig. S5 Growth curve of *E. coli* MG1655 strain harboring plasmids containing either pBK-TEM1-K73TAG or pBK-TEM1-K73TAG-Barnase(2TAA) in LB medium in the presence of 50 μg/mL kanamycin at 37 °C. The TEM1 gene was under control of a constitutive glnS promoter.

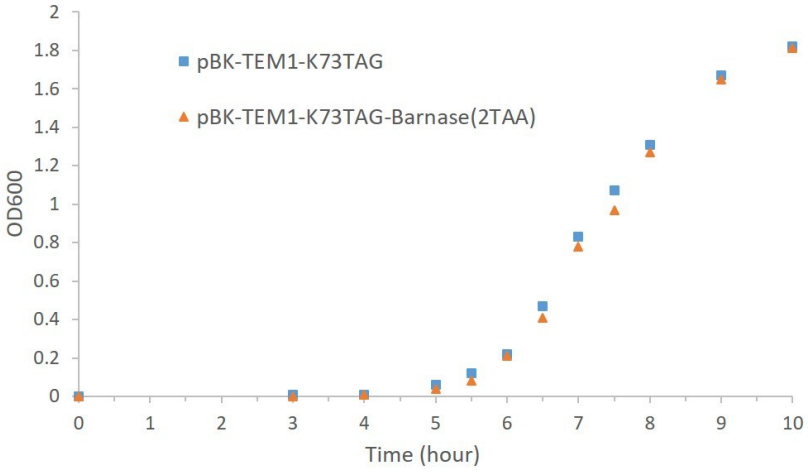

Fig. S6 Growth curve of *E. coli* MG1655 harboring TEM1-K73TAG-Barnase(2TAA) and AcKRS/$tRNA_{CUA}$ in LB medium in the presence of 100 μg/mL ampicillin, 25 μg/mL kanamycin, 25 μg/mL spectinomycin, and different amounts of AcK (0 – 5 mM) at 37 °C. 1 mM IPTG was added as inducer.