

Genetic incorporation of D-Lysine into diketoreductase in *Escherichia coli* cells **

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synthetase pairs, Stereoselectivity.

The genetic incorporation of unnatural amino acids with various side chains has provided a powerful tool to investigate protein structure and function. To date, more than 70 unnatural amino acids have been successfully incorporated into proteins by heterogeneous archaeal tRNA/aminoacyl-tRNA synthetase pairs in response to amber nonsense codon. While all genetically incorporated unnatural amino acids in cells are L-form, attempts to incorporate D-amino acids into proteins have only been limited to *in vitro* protein synthesis. For instance, D-Met and D-Phe were incorporated into proteins by modified ribosomes, and Hartman M.C. et al reported the synthesis of a number of peptides containing D-amino acids by utilizing *E. coli* aminoacyl-tRNA synthetases. However, these methods exhibited obvious limitations, such as low efficiency, poor yields and difficulty on scale-up. Consequently, it would be advantageous to incorporate D-amino acids into proteins in cells in order to study the roles of chirality in protein structure and function, and to probe the relationship between D-amino acids and pathogenetic progression. Typically, aminoacyl-tRNA synthetase and ribosome prefer L-amino acids for protein synthesis, but such specificity was suggested not to be absolute. In fact, it has been documented that a number of D-amino acids could be aminoacylated for the attachment to tRNA. On the

other hand, *Pyrococcus horikoshii* Lysyl-tRNA synthetase (*PhLysRS*) and tRNA_{CUA}^{Lys}

orthogonal pair could utilize a amber nonsense codon to expand the genetic code in *E. coli*,^[10] we docked the crystal structure of *PhLysRS*^[11] with D-Lys and L-Lys respectively. As shown in Figure S1a, the binding pocket of *PhLysRS* was able to well accommodate D-Lys with more hydrogen bondings than L-Lys (Figure S1b), suggesting that *PhLysRS* is possibly capable of aminoacylating D-Lys. Consequently, this orthogonal pair was employed in the present study to explore the possibility of incorporating D-Lys into proteins. Meanwhile, although D-Tyrosyl-tRNA deacylase

showed broad substrate specificity primarily responsible for the cleavage of the linkage between most D-amino acids and tRNA to avoid the incorporation of D-amino acids by ribosomal protein synthesis machinery, deletion of the *dtb* gene did not reduce the toxicity of *E. coli* K37 by various D-amino acids, including D-Lys,^[8] suggesting that the hydrolytic activity of D-Tyrosyl-tRNA deacylase towards D-Lysyl-tRNA might be very weak. In addition, the absence of specific D-Lysyl-tRNA deacylase activity in *E. coli* further increases the possibility that D-Lysyl-tRNA could be stable in cells and thus permitting D-Lys to be incorporated into proteins.

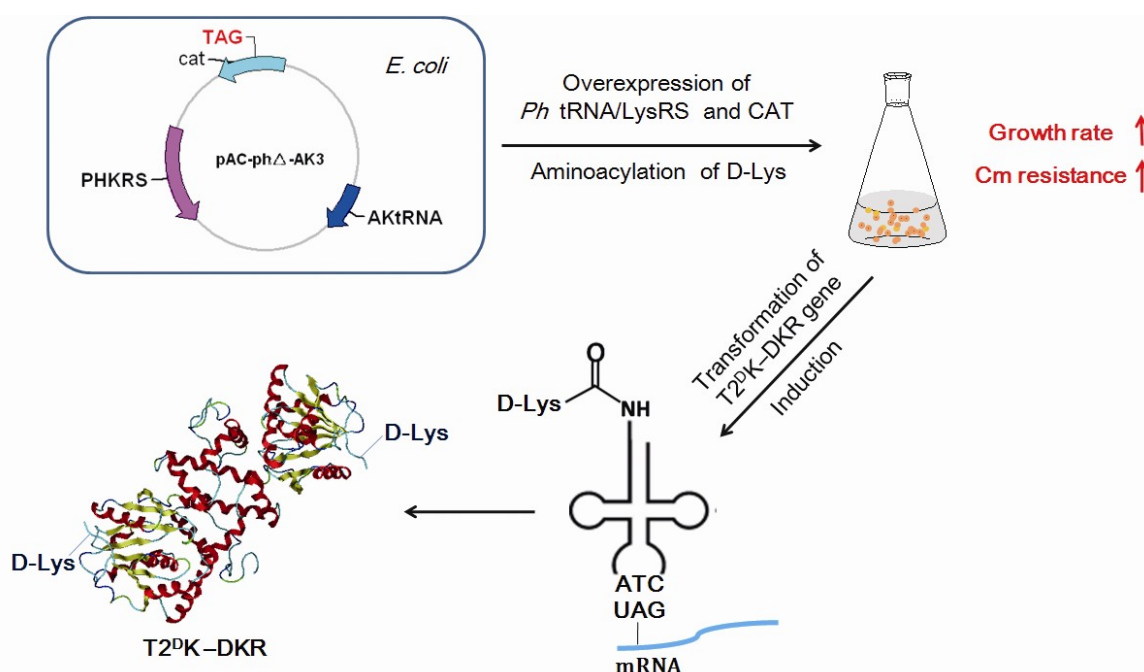


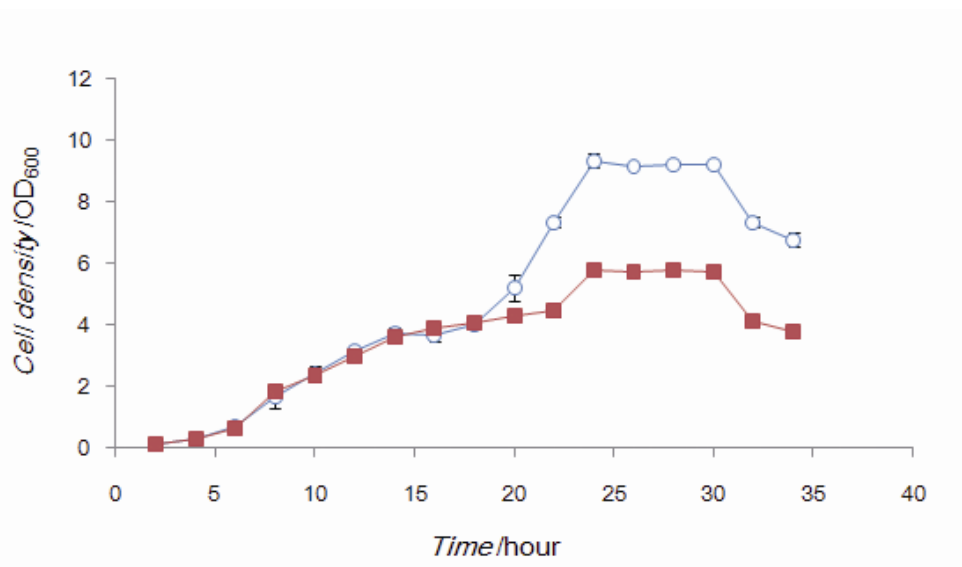
Figure 1. Strategy for incorporation of D-Lys into diketoreductase by *Ph* tRNA^{Lys}_{CUA}/LysRS orthogonal pair in *E. coli* cells.

First, the effects of D-Lys on the growth of *E. coli* BL21 cells with the expression of the orthogonal *Ph* tRNA^{Lys}_{CUA}/LysRS pair were investigated because D-Lys was normally toxic to *E. coli*.^[12] We constructed a plasmid pAC-phΔ-AK3 that encodes a

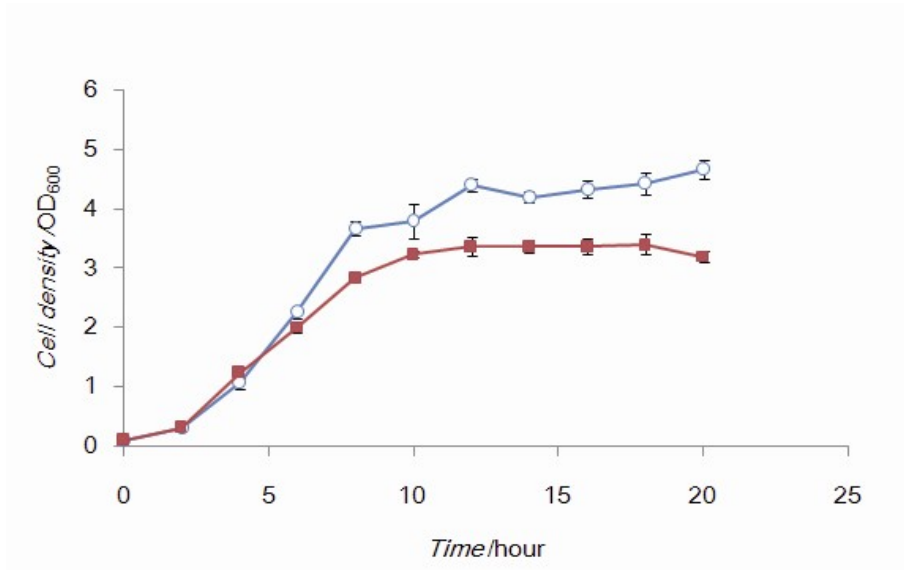
single copy of *Ph* LysRS gene and three copies of *Ph* tRNA^{Lys}_{CUA} gene to express the *Ph* tRNA^{Lys}_{CUA}/LysRS pair (Figure S2). Then, the pAC-ph Δ -AK3 plasmid was transformed into *E. coli* BL21 cells and an amber codon (TAG) was changed to encode lysine. Different from the strong inhibition of *E. coli* K37 by 5 mM D-Lys, the growth of *E. coli* cells harboring pAC-ph Δ -AK3 plasmid in M9 minimal medium supplemented with 5 mM D-Lys was surprisingly promoted (Figure 2a) compared to the control (Figure 2b). Figure 2c shows that D-Lys at different concentrations did not inhibit growth, indicating that D-Lys was not toxic to the transformed cells. Therefore, we postulated that the reduced toxicity of D-Lys might be due to the utilization of the excess amount of D-Lys by the *Ph* tRNA^{Lys}_{CUA}/LysRS pair to synthesize D-Lysyl-tRNA

tRNA^{Lys}_{CUA} in the cells.

a)



b)



c)

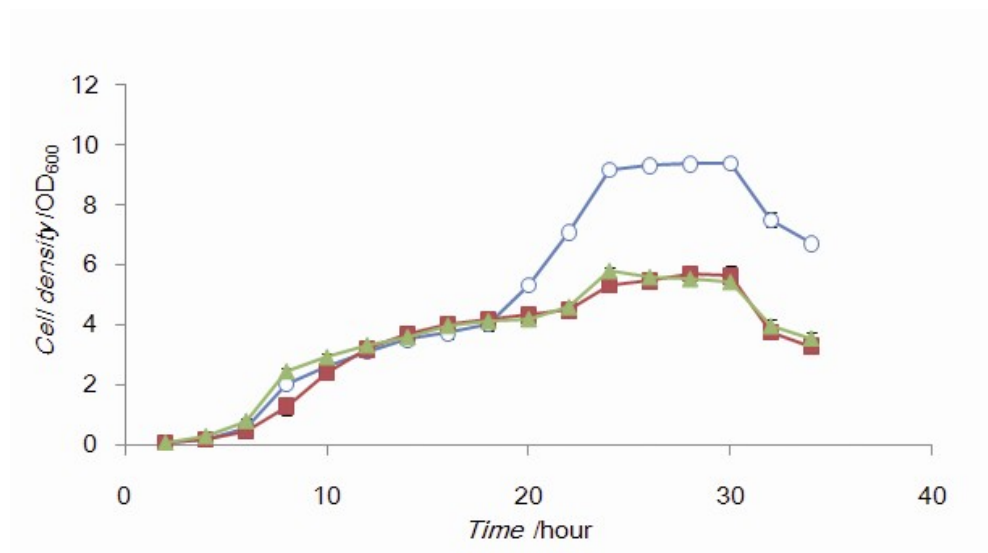


Figure 2. Effects of D-Lysine on the growth of engineered *E. coli* cells. a) Growth curves of *E. coli* BL21 (DE3) harboring pAC-ph Δ -AK3 in the absence and presence of D-Lys. (\square): M9 medium; (\circ): M9 medium plus 5 mM D-Lys. b) Growth curves of *E. coli* BL21 (DE3) cells in the absence and presence of D-Lys. (\circ): M9 medium. (\square): M9 medium plus 5 mM D-Lys. c) Effects of various concentrations of D-Lys on the growth curves of *E. coli* cells harboring pAC-ph Δ -AK3 plasmid. (\circ): M9 medium plus 3.6 mM D-Lys. (\square): M9 medium plus 2.4 mM D-Lys. (\triangle): M9 medium plus 1.2 mM D-Lys. Error bars represent standard deviations (n=3).

To verify our hypothesis, a chloramphenicol (Cm) reporting system (Figure S2a) was introduced to *E. coli* cells, in which the 112nd codon of chloramphenicol

acetyltransferase (CAT) was replaced by an amber codon.^[13] Since CAT cannot be expressed without the participation of *Ph* tRNA^{Lys}_{CUA}/LysRS pair, the expression of CAT would increase the resistance against Cm if D-Lys could be specifically utilized by *Ph* LysRS to aminoacylate with *Ph* tRNA^{Lys}_{CUA}. Consequently, we examined the effects of various concentrations of D-Lys on Cm resistance with *E. coli* cells harboring pAC-ph Δ -AK3. Indeed, all tested concentrations of D-Lys significantly enhanced Cm resistance as shown in Fig. 3. In the range of 1.2 to 3.6 mM D-Lys, the increase of Cm resistance was in a dose-dependent manner. These findings strongly supported that the *Ph* tRNA^{Lys}_{CUA}/LysRS pair could utilize D-Lys in the culture medium.

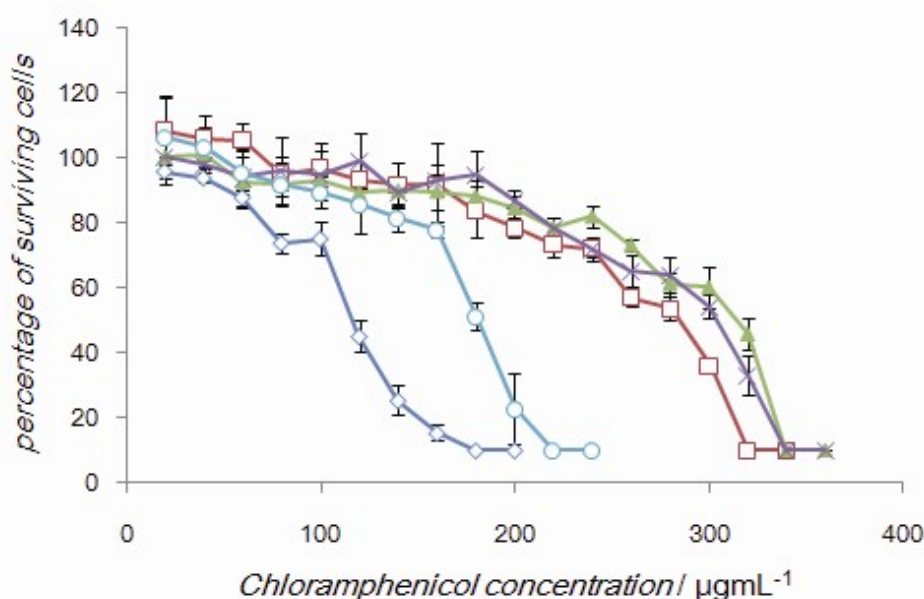


Figure 3. Effects of various concentrations of D-Lys on Cm resistance of *E. coli* cells harboring pAC-ph Δ -AK3 plasmid. (◇): M9 medium (IC₅₀=120 µg.mL⁻¹); (○): M9 medium plus 1.2 mM D-Lys (IC₅₀=180 µg.mL⁻¹); (□): M9 medium plus 2.4 mM D-Lys (IC₅₀= 280 µg.mL⁻¹); (×): M9 medium plus 5 mM D-Lys (IC₅₀= 320 µg.mL⁻¹); (△): M9 medium plus 3.6 mM D-Lys (IC₅₀= 340 µg.mL⁻¹). Error bars represent standard deviations (n=3).

To further examine whether D-Lys can be incorporated into a foreign protein in the cells, we chose diketoreductase (DKR) that has been extensively studied in our laboratory as a target protein. Because the Thr residue at the second position of DKR is located in the N-terminus^[15] and any mutations at this position do not influence enzyme activity (data not shown), this Thr residue was selected for switching to D-Lys. Thus, we created a pETduet-T2^DK-*dkr* construct (Figure S2b), in which the 2nd codon of DKR gene was mutated from ACC to TAG. After co-transforming pETduet-T2^DK-*dkr* and pAC-ph Δ -AK3 into *E. coli* BL21, the resulting cells were cultured in M9 minimal medium containing 3.6 mM D-Lys and the mutant designated as T2^DK-DKR was expressed by IPTG induction. Meanwhile, the wild-type *dkr* gene co-transformed with the same orthogonal pair was expressed under the same conditions. As expected from the suppression of tRNA, the expression level of T2^DK-DKR mutant was markedly lower compared to wild-type (Figure S3). The expressed T2^DK-DKR was purified by two-step chromatography to homogeneity (Figure S4). Then, purified T2^DK-DKR protein was hydrolyzed by acid, and the dried hydrolysate was derivatized and analyzed by chiral HPLC. Compared with the HPLC chromatograms of D-Lys and L-Lys derivatives, the derivative of D-Lys from T2^DK-DKR hydrolysate at 113.3 min was clearly detected and quantified as shown in Figure 4. After the same procedures of purification, hydrolysis and derivatization, wild-type DKR did not produce the peak from the derivatization for D-Lys. Because T2^DK-DKR contains 20 Lys residues and theoretically the ratio of D- vs. L-stereoisomer should be 5.26 % if a D-Lys was completely incorporated into the protein, our experimental ratio was 5.87 \pm 0.18% (n=3) after quantifying the peak areas in HPLC chromatograms. This incorporation ratio confirmed that only one D-Lys was selectively incorporated into T2^DK-DKR protein. Although it is unclear how the protein synthesis exactly takes place, the remarkable increase of Cm resistance in the presence of D-Lys may provide a basis for increased stereoselectivity of *Ph* tRNA_{CUA}^{Lys}/LysRS pair towards D-Lys,

which is in an agreement with previous reports on the substrate specificity.^[16]

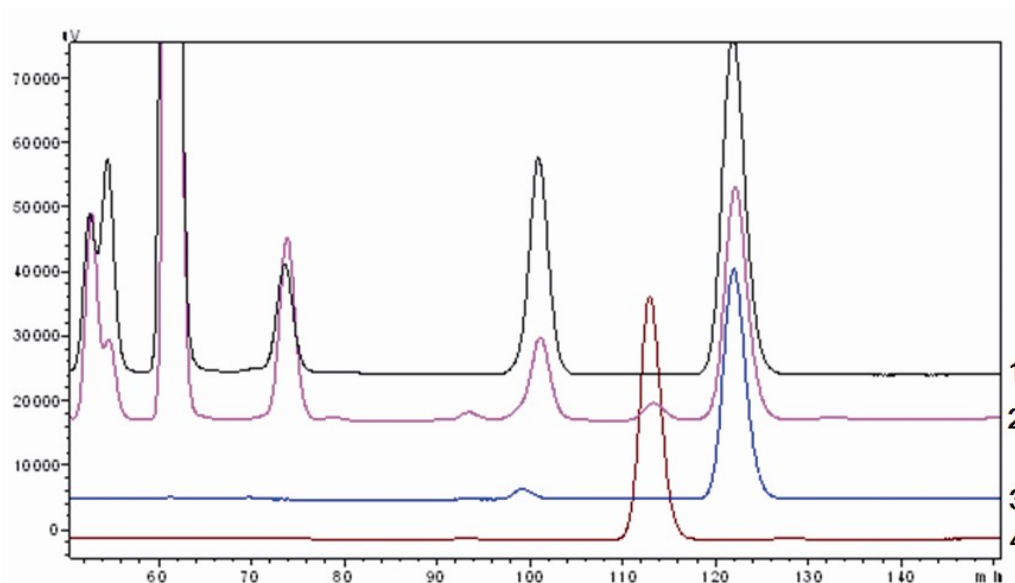


Figure 4. Chiral HPLC analysis of the derivatives of hydrolyzates and Lysine stereoisomers. 1: Wild-type DKR; 2: T2^DK mutant; 3: L-Lys; 4: D-Lys. The retention times of D-Lys and L-Lys derivatives were 113.3 and 122.2 min respectively.

In conclusion, we have demonstrated that the expression of *Ph* tRNA^{Lys}_{CUA}/LysRS pair could reduce the toxicity of D-Lys to *E. coli* cells. Increase of Cm resistance also suggested that *Ph* LysRS could be highly stereoselective towards D-Lys *in vivo* when excess amount of D-Lys is present in the culture medium. Expression of T2^DK-DKR mutant and analysis of D-Lys derivative by chiral HPLC further confirmed the genetic incorporation of D-Lys. In addition to D-Lys, the present strategy may be applicable to other D-amino acids by the genetic manipulation of tRNA/aminoacyl-tRNA synthetase orthogonal pairs. Moreover, the genetic incorporation of D-amino acids may provide a new tool for the investigation of protein structure and function.

Experimental Section

Briefly, all engineered *E. coli* cells grew in the presence of respective antibiotics. Cell growth rates and chloramphenicol resistance were determined by measuring OD₆₀₀ at various time points. Plasmids of pAC-ph Δ -AK3 and pETDuet-

T2^DK-*dkr* were constructed and transformed to *E. coli* BL21 (DE3) cells. Subsequently, T2^DK-DKR mutant was expressed by IPTG induction and purified by Nickel affinity and anion-exchange chromatography. Then, the purified T2^DK-DKR mutant was hydrolyzed by 10 N HCl and derivatized with 4-fluoro-7-nitro-2, 1, 3-benzoxadiazole, and the derivatives of D-Lys and L-Lys were analyzed by HPLC on a Chiralcel OD-RH column with a gradient elution.

(Detailed excremental procedures can be found in the Supporting information.)

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Supporting Information

1. Experimental details

2. Supplementary figures

3. Supplementary tables

4. Supplementary references

1. Experimental details

1.1 Materials

4-Fluoro-7-nitro-2, 1, 3-benzoxadiazole (NBD-F) was purchased from Sigma (Sigma, USA). D-Lysine monohydrochloride was purchased from Qiude Biochemical Engineering Co (Qiude, China). *Pyrococcus horikoshii* ATCC 700860 and pACYC184 plasmid in *E. coli* ATCC 37033 were obtained from American Type Culture Collection (ATCC, USA). lpp-AKtRNA-rrnC gene, GlnRS promoter and terminator genes were synthesized according to the patent ^[1] and ligated to pMD-18T vector. pMD-18T and pETDuet-1 vectors were obtained from TaKaRa (Japan) and Novagen (USA), respectively. All restriction endonucleases and T4 DNA ligase were purchased from Fermentas (USA) and TakaRa (Japan). HPLC analysis was performed on Shimadzu 2010A HT with UV detector (Japan). All other reagents were purchased from Sigma Co. (USA).

1.2 Sources of LysRS/ tRNA^{Lys}_{CUA} orthogonal pair

According to previous report and patent, ^[1, 2] tRNA^{Lys}_{CUA} from *Pyrococcus horikoshii* ATCC 700860 was synthesized by GenScript Co. (China), and Lysyl-tRNA synthetase was amplified from the same strain by PCR as described in 1.4.

1.3 Molecular docking of PhLysRS with D-Lys and L-Lys

D-Lys and L-Lys were docked to active site of crystal structure of PhLysRS (PDB: 1lrx) using Discovery studio 2.5 (DS2.5). The sdf files of D- and L-Lys were obtained from the PubComp library of the NCBI database (<http://www.ncbi>).

nlm.nih.gov/pccompound). The crystal structure of *PhLysRS* was from the PDB database (<http://www.pdb.org>). The Libdock algorithm and default values of DS2.5 were used to dock *PhLysRS* with D-Lys and L-Lys, respectively. The software generated 100 poses for D-Lys and L-Lys, which were numbered according to Libdock scores. Then, model structures were selected based on the Libdock scores (>50) and the maximal number of hydrogen bonding. The binding energy between enzyme and substrate was subsequently calculated.

1.4 Construction and transformation of pAC-ph Δ -AK3 and pETDuet-T2^DK-*dkr*.

P. horikoshii ATCC 700860 was anaerobically cultivated in ATCC medium 1915 at 95°C for 7 days. The genomic DNA of *P. horikoshii* ATCC 700860 was extracted with TaKaRa MiniBEST DNA Extraction Kit (Japan). The *Ph* Lysyl-tRNA synthetase gene was amplified using genomic DNA from *P. horikoshii* ATCC 700860 as a template and primers with *KpnI* and *PstI* restriction sites (Table S1). The created *KpnI-PstI* fragment was ligated to pMD-18T vector, and the resulting plasmid was designated as pMD-18T-*phkrs*. Next, to construct the pAC-*phkrs*, pMD-18T-*phkrs* was digested with *KpnI* and *PstI*, and the resulting *phkrs* fragment was purified and ligated to pACYC184 digested with the same restriction enzymes. *lpp-AKtRNA-rrnC* gene was amplified for three times using pMD-18T-*trna* as a template and the primers consisting of three pairs of restriction sites *XbaI*; *BamHI*; *EcoRI*, *HindIII* respectively (Table S1). The three different *aktRNA* gene fragments were digested with *XbaI*, *BamHI*; *BamHI*, *EcoRI*; *EcoRI*, *HindIII* respectively and ligated to pETDute-1 digested with the same restriction enzymes. The resulting plasmid was named as pETDuet-AK3. Subsequently, to construct the pAC-ph Δ -AK3, pETDuet-Ak3 and pAC-*phkrs* was digested with *XbaI* and *HindIII* respectively and the resulting AKtRNA3 fragments were ligated with pAC-*phkrs* fragments.

dkr-TAG gene fragment was amplified using pET22b (+)-*dkr* as a template and the primers with *BamHI* and *HindIII* restriction sites that contain an amber codon (Table S1). The *BamHI-HindIII* fragment with TAG was ligated to pMD-18T vector. Subsequently, to construct plasmid pETDuet-T2^DK-*dkr*, pMD-18T-*dkr*-TAG was

digested with *Bam*HI and *Hind*III. The resulting *dkr*-TAG fragment was purified and ligated to pETDuet-1 vector digested with the same restriction enzymes.

To co-transform pAC-ph Δ -AK3 and pETDuet-T2^DK-*dkr* into *E. coli* cells, 100 ng pAC-ph Δ -AK3 and 80 ng pETDuet-T2^DK-*dkr* was mixed with 100 μ l competent *E. coli* BL21 (DE3) cells. The mixture was placed on ice for 30 min, and then heated for 100 s at 42°C. Subsequently, the mixture was diluted by 800 μ l LB broth and incubated at 37°C for 50 min; finally, the diluted mixture was spread to LB agar plate and cultured at 37°C for overnight. Clones were picked and culture at 5 ml LB broth containing 20 μ g/ml chloramphenicol, 4 μ g/ml tetracycline and 40 μ g/ml ampicillin.

1.5 Enzyme assay for DKR and T2^DK-DKR mutant

According to the literature,^[3] spectrophotometric method was used to determine the DKR and T2^DK-DKR mutant activity on a UV-1700 array spectrophotometer (Japan) whose cell compartment was maintained at 40°C during the measurements of absorbance change at 340 nm for the oxidation of NADH. Assay mixture contained 0.15 mM NADH, 0.25 mM ethyl-6-(benzyloxy)-3, 5-dioxo- hexanoate, 5–50 mg DKR or T2^DK-DKR mutant and 0.1 M potassium phosphate buffer (pH 6.0) in a final volume of 1.0 ml. The decrease of absorbance at 340 nm was monitored by the addition of enzyme. The reaction was linear in 2 min. For all assays, enzyme activity was defined as one unit representing the oxidation of one μ mole of NADH per minute per milligram protein.

1.6 Expression and purification of T2^DK-DKR mutant.

E. coli BL21 (DE3) cells harboring pAC-ph Δ -AK3 and pETDuet-T2^DK-*dkr* were cultivated overnight in M9 medium (50 ml) containing 20 μ g/ml chloramphenicol, 4 μ g/ml tetracycline and 40 μ g/ml ampicillin at 37°C, then the cultures were diluted into fresh M9 medium containing 3.6 mM D-Lys with an OD₆₀₀ of 0.30 and incubated to OD₆₀₀ of 0.6, T2^DK-DKR mutant was expressed by addition of 200 mM IPTG at 37 °C, 250 rpm for 20 h. Then, *E. coli* cells in 200 ml broth were harvested by centrifugation at 8000 x *g* for 10 min. 5g cells was diluted in 25 ml 50 mM sodium phosphate buffer (pH8.0) and disrupted by high-pressure cell-disruption systems

(UK). The lysate was centrifuged for 30 min at 13,500 x *g* at 4°C, and 25mL supernatant was loaded to Ni-NTA column (2.0 cm × 20 cm, Novagen, USA) and static absorbed for overnight at 4 °C. The column was successively washed with 50 mM sodium phosphate buffer (pH 8.0) containing 10 and 50 mM imidazole for 10 and 5 column volumes respectively; then, the T^{2D}K-DKR mutant protein was eluted with 100 mM imidazole in the same buffer. The eluate was concentrated with a centrifugal filter device (Millipore Corp., USA). The concentrated proteins was then applied onto a DEAE-Sepharose (GE Healthcare Biosciences, USA) column (2.0 cm × 5.0 cm) equilibrated with 50 mM sodium phosphate buffer (pH 8.0) containing 1 mM DTT and 5% glycerol. After equilibrium with the buffer, the column was eluted with 100 ml of a linear gradient (0–0.5 M NaCl). Fractions with 3 ml each were collected at a flow rate of 1.5 ml/min. Active fractions were pooled, concentrated and desalted using the centrifugal filter device.

1.7 Hydrolysis of DKR and T^{2D}K-DKR and derivatization of the hydrolyzates.

Three mg purified T^{2D}K-DKR mutant and wild-type DKR were hydrolyzed in 30 ml 10 N HCl at 110 °C for 20 h. Next, the hydrolyzates were dried up by vacuum evaporation, subsequently, the dried hydrolyzates were dissolved in 500 µl of 50 mM sodium phosphate buffer (pH8.0) and adjusted to pH 8.0 using 10 N NaOH.

Derivatization of the hydrolyzates and lysine stereoisomers was performed according to the literature^[4]. Briefly, 50 µl samples was mixed with 30 µl of 50 mM NBD-F in acetonitrile and heated at 60 °C for 5 min to complete the reaction. Then, 920 µl of 1% acetic acid in methanol was added to the solution to stop the reaction.

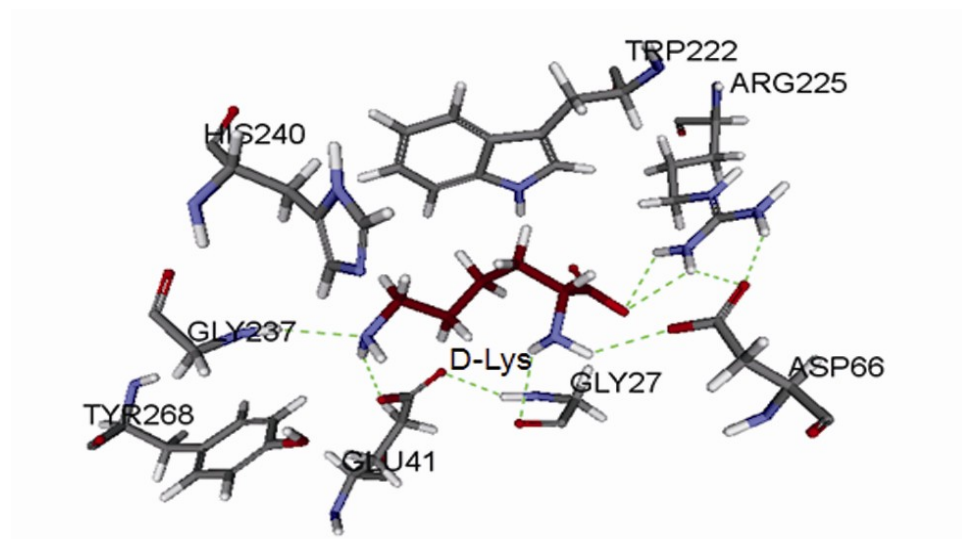
1.8 Chiral HPLC analysis

Shimadzu SCL-2010A with autosampler was used for the analysis. The HPLC analysis was performed on a Chiralcel OD-RH column (5 µm, 150×4.6 mm) at 10 °C with an injection volume of 20 µl and a flow rate of 0.3 ml/min. Mobile phases consisted of 0.1 % TFA aqueous solution (A) and acetonitrile (B). Mobile phases were degassed by ultrasonic vacuum for 30 min. Elution was carried out with a gradient of 35–40% B in 150 min, and then 40% B was kept for additional 10 min. Detection was

recorded at wavelength of 470 nm. All samples were filtered with 0.22 μm micropore filter (Millipore Corp., USA).

2. Supplementary figures

a)



b)

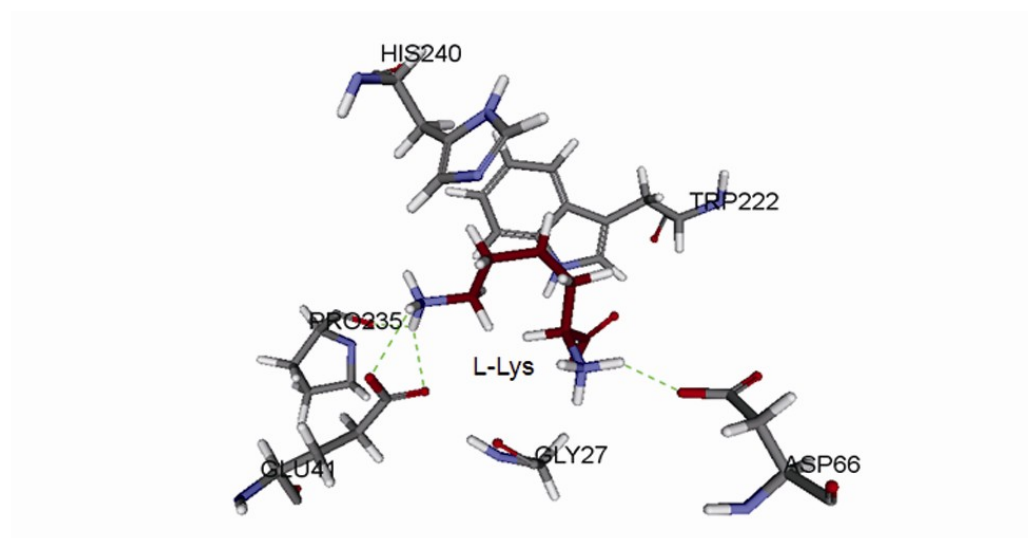
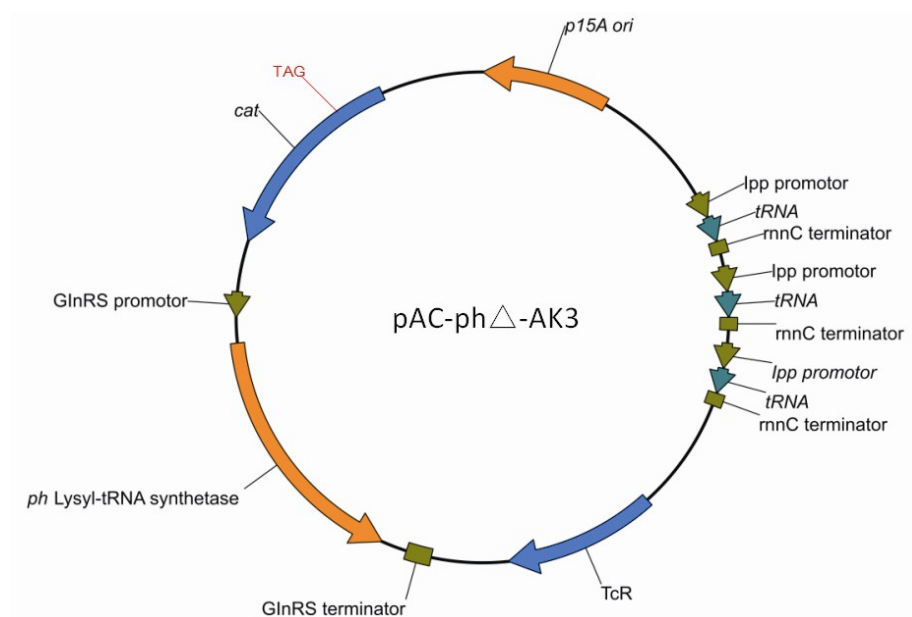


Figure S1 Schematic views of the interactions between *PhLysRS* and D-Lys and L-Lys. Lys (in red) and active site residues of *PhLysRS* are indicated as sticks. The carbon, nitrogen, oxygen and hydrogen atoms are shown in gray, blue, red and white respectively. Hydrogen bondings between Lys and active site residues are displayed with green dashed lines. a) Interactions between D-Lys and active site residues of *PhLysRS*. The carboxyl group of D-Lys forms two hydrogen bondings with the

guanido group of Arg225, and α -amino group of D-Lys produces two hydrogen bondings with the carboxyl groups of Asp66 and Gly27 respectively. The ϵ -amino group of D-Lys forms two hydrogen bondings with the carboxyl group of Glu41 and the amino group of Gly237. The binding energy between *PhLysRS* and D-Lys was calculated to be -13.629 kJ/mol. b) Interactions between L-Lys and active site residues of *PhLysRS*. The α -amino group of L-Lys forms a hydrogen bonding with the carboxyl group of Asp66, and the ϵ -amino group of L-Lys produces two hydrogen bondings with the carboxyl group of Glu41. The binding energy between *PhLysRS* and L-Lys was calculated to be -14.027 kJ/mol.

a)



b)

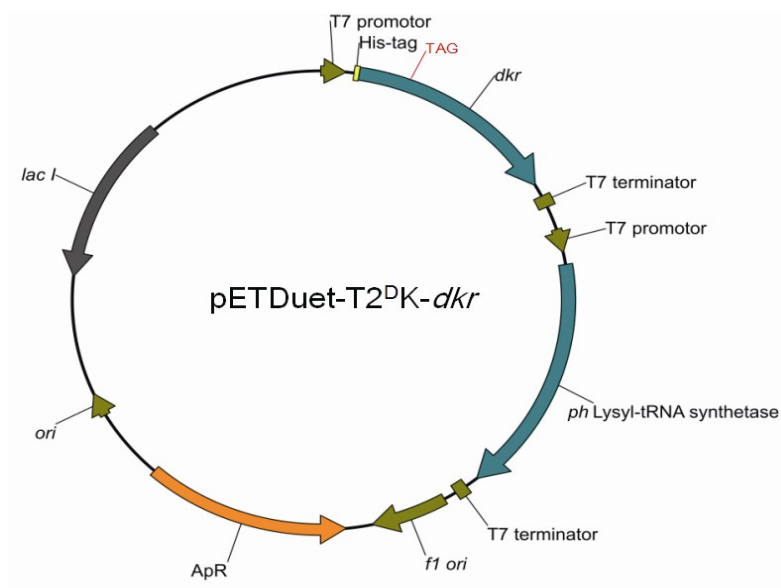


Figure S2. Constructed plasmid map. a. pAC-ph Δ -AK3. b. pETDuet-T2^DK-dkr.

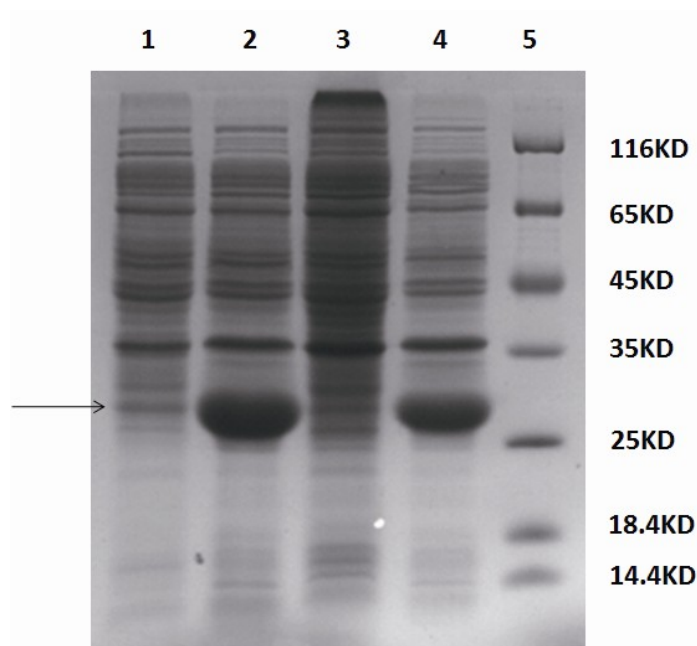


Figure S3. 12% SDS-PAGE for protein expression of T2^DK-DKR mutant and wild type DKR in recombinant *E. coli* cells after induction. Lane 1, Soluble fraction of T2^DK-DKR mutant; Lane 2, Soluble fraction of wild type DKR; Lane 3, Whole cells of T2^DK-DKR mutant; Lane 4, Whole cells of wild type DKR; Lane 5, Markers for protein molecular weight. Arrow indicates the target protein.

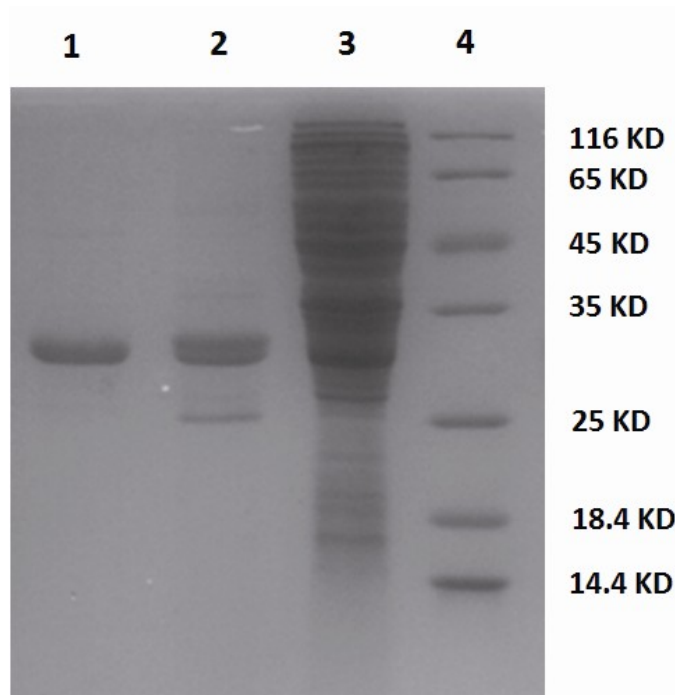


Figure S4. 12% SDS-PAGE for the purification of T2^DK-DKR mutant. Lane 1, Purified T2^DK-DKR protein after DEAE- Sepharose column; Lane 2, T2^DK-DKR protein after Ni-NTA column; Lane 3, Crude extract of T2^DK-DKR mutant; Lane 4, Markers for protein molecular weight.

3. Supplementary tables

Table S1 Primers used in this experiment.

Primer	5' to 3'	Restriction site
PHKRSU:	5'-CGGGGT <u>ACCATGGTTCATTGGGCCGATTATATTG</u> -3'	<i>KpnI</i>
PHKRSL:	5'-ATACTGCAGTTATGAAAGCTCGTAAGTCCTCCTT-3'	<i>PstI</i>
AKxbaU:	5'-ATATCTAGAGGTAAGATCTCGAACGATCAAAAATAAGTGC-3'	<i>XbaI</i>
AKBamL:	5'-ATAGGATCCGGTTGCATGCAAAAAAATCCTTAGCTTTTCG-3'	<i>BamHI</i>
AKBamU:	5'-ATAGGATCCGGTAAGATCTCGAACGATCAAAAATAAGTGC-3'	<i>BamHI</i>
AKEcoRL:	5'-CCGGAATTCGGTTGCATGCAAAAAAATCCTTAGCTTTTCG-3'	<i>EcoRI</i>
AKEcoRU:	5'-CCGGAATTCGGTAAGATCTCGAACGATCAAAAATAAGTGC-3'	<i>EcoRI</i>
AKHindL:	5'-CCCAAGCTTGGTTGCATGCAAAAAAATCCTTAGCTTTTCG-3'	<i>HindIII</i>
DKRBamHI:	5'-CGCGGATCCATGTAGGGCATCACGAATGTCACCGTTCT-3'	<i>BamHI</i>
DKRHindIII:	5'-CCCAAGCTTTCAGTACCGGTAGAAGCCCTCGCCGCTCGC-3'	<i>HindIII</i>

Underlines represent the sequences of restriction sites.

4. Supplementary references

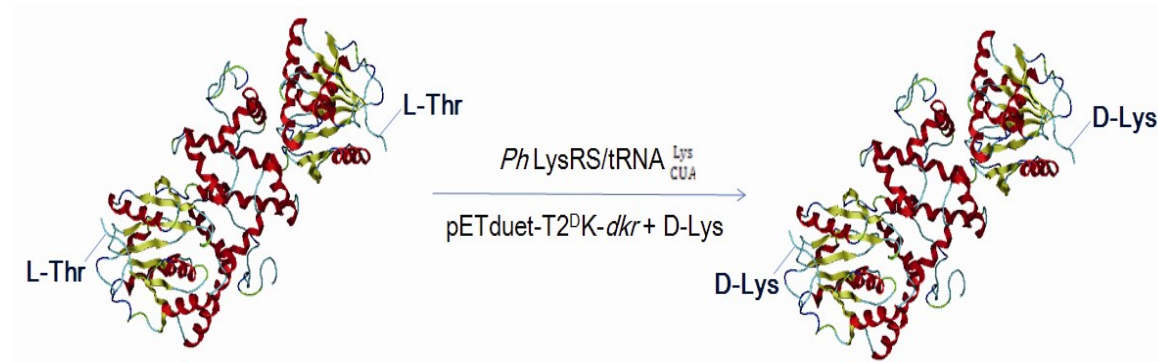
[1] J. C. Anderson, N. Wu, S. W. Santoro, V. Lakshman, D. S. King, P. G. Schultz, An expanded genetic code with a functional quadruplet codon. *Proc. Natl. Acad. Sci. U S A* 2004, *101*, 7566.

[2] J. C. Anderson, N. Wu, S. Santoro, P. G. Schultz, Compositions of orthogonal lysyl-tRNA and aminoacyl-tRNA synthetase pairs. US patent, 2006, US7575895B2.

[3] X. Wu, N. Liu, Y. He, Y. Chen, *Acta. Biochim. Biophys. Sin (Shanghai)* 2009, *41*, 163.

[4].T. Miyamoto, M. Sekine, T. Ogawa, M. Hidaka, H. Homma, H. Masaki, *Amino Acids* 2010, *38*, 1377.

Biographical sketch:



D-Lysine has been genetically introduced into diketoreductase in *E. coli* cells by

utilization of an orthogonal *Ph* tRNA^{Lys}_{CUA}/Lysyl-tRNA synthetase pair.