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SHORT COMMUNICATION

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

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Abstract *Pyrococcus horikoshii* lysyl-tRNA synthetase/tRNA orthogonal pair exhibited high selectivity towards D-lysine in the presence of excess amount of D-lysine. Based on the observation, this orthogonal pair was employed to encode D-lysine, and D-lysine was site-specifically incorporated into the diketoreductase in *E. coli* cells.

Keywords Genetic incorporation · D-Lysine · tRNA/aminoacyl-tRNA synthetase orthogonal pair · Stereoselectivity

Introduction

The genetic incorporation of unnatural amino acids with various side chains has provided a powerful tool to investigate the structure and function of proteins (Wang et al. 2001). 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 (Liu et al. 2010). While all genetically incorporated unnatural amino acids in cells are L-form, attempts to incorporate D-amino acids into proteins were only limited to in vitro protein synthesis prior to the present study. For instance, D-Met and D-Phe were incorporated into proteins by modified ribosomes (Dedkova et al. 2003), and Hartman et al. (2006) 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 to study the roles of chirality in protein structure and function (Krause et al. 2000), and to probe the relationship between D-amino acids and pathogenetic progression (Fujii et al. 2011). Typically, aminoacyl-tRNA synthetase and ribosome prefer L-amino acids for protein synthesis, but such specificity was suggested not to be absolute (Bhuta et al. 1981; Jakubowski 1999; Soutourina et al. 2000). In fact, it has been documented that a number of D-amino acids could be aminoacylated for the attachment to tRNA (Calendar and Berg 1966; Takayama et al. 2005), especially mutated L-lysyl-tRNA synthetase from *Bacillus cereus* was able to aminoacylate D-β-Lys (Gilreath et al. 2011). In addition, it was reported that evolved aminoacyl-tRNA synthetases exhibited broad specificity towards structurally similar substrates and were able to recognize multiple unnatural amino acids (Young et al. 2011). Meanwhile, *Pyrococcus horikoshii* lysyl-tRNA synthetase (*Ph*LysRS) and $tRNA^{Lys}_{CUS}$ orthogonal pair could utilize an amber nonsense codon to expand the genetic code in *E. coli* (Anderson et al. 2004). Therefore, this orthogonal pair was employed in the present study to explore the possibility of incorporating D-Lys into proteins. After co-transforming the *Ph*LysRS and $tRNA^{Lys}_{CUS}$ orthogonal pair and *dkr* gene with an amber codon in *E. coli*, D-Lys was characterized to be successfully incorporated into diketoreductase (DKR).

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Materials and methods

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 (USA). Suppressor tRNA with lpp promoter and rrnC terminator (lpp-AKtRNA-rrnC), GlnRS promoter and terminator genes were synthesized according to the patent (Anderson et al. 2006) and ligated to pMD-18T vector and the resulting plasmids were designated as pMD-18T-*trna* and pMD-18T-*glnrspt*. All restriction endonucleases and T4 DNA ligase were purchased from Fermentas (USA) and TakaRa (Japan). All other regents were purchased from Sigma Co. (USA).

Sources of the LysRS/tRNA$^{Lys}_{CUS}$ orthogonal pair

According to previous report and patent (Anderson et al. 2004, 2006), tRNA$^{Lys}_{CUS}$ from *Pyrococcus horikoshii* ATCC 700860 was synthesized by GenScript Co. (China), and Lysyl-tRNA synthetase was amplified from the same strain by PCR.

Molecular docking

D-Lys and L-Lys were docked to the active site of the crystal structure of *Ph*LysRS (PDB: 1IRX) 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 Libdock algorithm and default values of DS2.5 were used for docking, and 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.

Construction and transformation of pAC-phΔ-AK3 and pETDuet-T2^{D}K-*dkr*

Ph Lysyl-tRNA synthetase gene was amplified using genomic DNA from *P. horikoshii* ATCC 700860 as a template and primers with *Kpn*I *and Pst*I restriction sites (Supplementary Table 1). The created *Kpn*I–*Pst*I fragment was ligated to pMD-18T vector. The pMD-18T-*phkrs* was digested with *Kpn*I *and Pst*I, and the resulting *phkrs* fragment was purified, and ligated to pACYC184. 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 *Xba*I; *Bam*HI; *Eco*RI; *Hin*dIII, respectively (Supplementary Table 1).The three different *ak*tRNA gene fragments were digested with *Xba*I, *Bam*HI; *Bam*HI, *Eco*RI; *Eco*RI, *Hin*dIII, respectively, and ligated to pETDute-1 to result in pETDuet-AK3. Subsequently, pETDuet-Ak3 and pAC-*phkrs* was digested with *Xba*I and *Hin*dIII and the resulting *ak*tRNA3 fragments were ligated with pAC-*phkrs* to obtain pAC-phΔ-AK3 (the plasmid harboring the genes of *Ph*LysRS/tRNA$^{Lys}_{CUS}$ orthogonal pair, see Supplementary Fig. 2a).

dkr-TAG fragment (*dkr* gene with an amber codon at 2nd site) was amplified using pET22b (+)-*dkr* as a template and the primers with *Bam*HI *and Hin*dIII restriction sites that contain an amber codon (Supplementary Table 1). The *Bam*HI–*Hin*dIII fragment with TAG was ligated to pMD-18T vector. Subsequently, plasmid pETDuet-T2^{D}K-*dkr* (pETDuet harboring the amber mutated *dkr* gene, Supplementary Fig. 2b) was obtained by ligation of *dkr*-TAG fragment and pETDuet-1 vector.

Co-transformation of pAC-phΔ-AK3 and pETDuet-T2^{D}K-*dkr* into *E. coli* BL21 (DE3) was achieved by mixing pAC-phΔ-AK3 and pETDuet-T2^{D}K-*dkr*. Then, after incubation of the cells in LB broth at 37 °C for 50 min, diluted mixture was cultured in LB agar plate at 37 °C overnight. Clones were picked and cultured at 5 ml LB broth containing 20 μg ml^{-1} chloramphenicol, 4 μg ml^{-1} tetracycline, and 40 μg ml^{-1} ampicillin.

Enzyme assay

A spectrophotometric method was used to determine the activity of DKR and T2^{D}K-DKR mutant according to the literature (Wu et al. 2009).

Expression and purification of T2^{D}K-DKR mutant

Escherichia coli BL21 (DE3) cells harboring pAC-phΔ-AK3 and pETDuet-T2^{D}K-*dkr* were cultivated overnight in M9 medium (50 ml) containing 20 μg ml^{-1} chloramphenicol, 4 μg ml^{-1} tetracycline and 40 μg ml^{-1} ampicillin at 37 °C; then the cultures were diluted into fresh M9 medium containing 3.6 mM D-Lys to OD_{600} of 0.3 and incubated to OD_{600} of 0.6; T2^{D}K-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 8,000×*g* for 10 min. Five grams cells was diluted in 25 ml 50 mM sodium phosphate buffer (pH 8.0) and disrupted by high-pressure cell-disruption systems (UK). The lysate was centrifuged for 30 min at 13,500×*g* at 4 °C and 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 T2^{D}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^{-1}. Active fractions were pooled, concentrated and desalted using the centrifugal filter device.

Hydrolysis of DKR and T2^{D}K-DKR and derivatization of the hydrolyzates

Three milligrams purified T2^{D}K-DKR mutant and wild-type DKR was 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 (pH 8.0) and adjusted to pH 8.0 using 10 N NaOH.

Derivatization of the hydrolyzates and lysine stereoisomers was performed according to the literature (Miyamoto et al. 2010). Briefly, 50 µl samples were 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.

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^{-1}. 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 Co.).

Trypsin digestion and mass spectrometry

First, the bands of T2^{D}K-DKR and WT-DKR were cut from SDS-PAGE. Three hundred microlitres of 100 mM NH_4HCO_3 containing 30 %ACN was added to decolorize the gel. Then, the gel-embedded proteins were reduced and alkylated using 100 µL 100 mM NH_4HCO_3 containing 10 mM DTT at 56 °C for 30 min and 30 µL 200 mM iodoacetamide in dark for 20 min, respectively. Subsequently, the proteins were digested with trypsin at 37 °C for 20 h. MALDI-TOF mass spectrometry with a positive ion mode was performed on Applied Biosystem 4800 series (USA). Data were collected between 800 and 4000 *m*/*z* and outputted by Data Explorer V4.5.

Results and discussion

Molecular docking of *Ph*LysRS with D-Lys and L-Lys

The crystal structure of *Ph*LysRS (Terada et al. 2002) was docked with D-Lys and L-Lys, respectively. As shown in Supplementary Fig. 1a, the binding packet of *Ph*LysRS was able to well accommodate D-Lys with more hydrogen bondings than L-Lys (Supplementary Fig. 1b), suggesting that *Ph*LysRS, in addition to L-Lys, is able to aminoacylate D-Lys.

D-Lysine did not inhibit the growth of *E. coli* cells harboring pAC-phΔ-AK3 plasmid

The effects of D-Lys on the growth of *E. coli* BL21 cells with the expression of the orthogonal *Ph* $tRNA^{Lys}_{CUS}$/LysRS pair were investigated because D-Lys was normally toxic to *E. coli* (Soutourina et al. 2004). We constructed a plasmid pAC-phΔ-AK3 that encodes a single copy of *Ph* LysRS gene and three copies of the *Ph* $tRNA^{Lys}_{CUS}$ gene to express the *Ph* $tRNA^{Lys}_{CUS}$/LysRS pair (Supplementary Fig. 2a). Then, the pAC-phΔ-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 (Soutourina et al. 2004), the growth of *E. coli* cells harboring pAC-phΔ-AK3 plasmid in M9 minimal medium supplemented with 5 mM D-Lys was surprisingly promoted (Figs. 1, 2a) compared with the control (Fig. 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, the reduced toxicity of D-Lys might be due to the utilization of the excess amount of D-Lys by the *Ph* $tRNA^{Lys}_{CUS}$/LysRS pair to synthesize D-lysyl-$tRNA^{Lys}_{CUS}$ and the decrease of interference from D-Lys to the endogenous tRNA synthetase in the cells.

D-Lysine increased the Cm resistance of *E. coli* cells harboring pAC-phΔ-AK3 plasmid

Because chloramphenicol acetyltransferase (CAT) cannot be expressed without the participation of *Ph* $tRNA^{Lys}_{CUS}$/LysRS pair, the expression of CAT would increase the

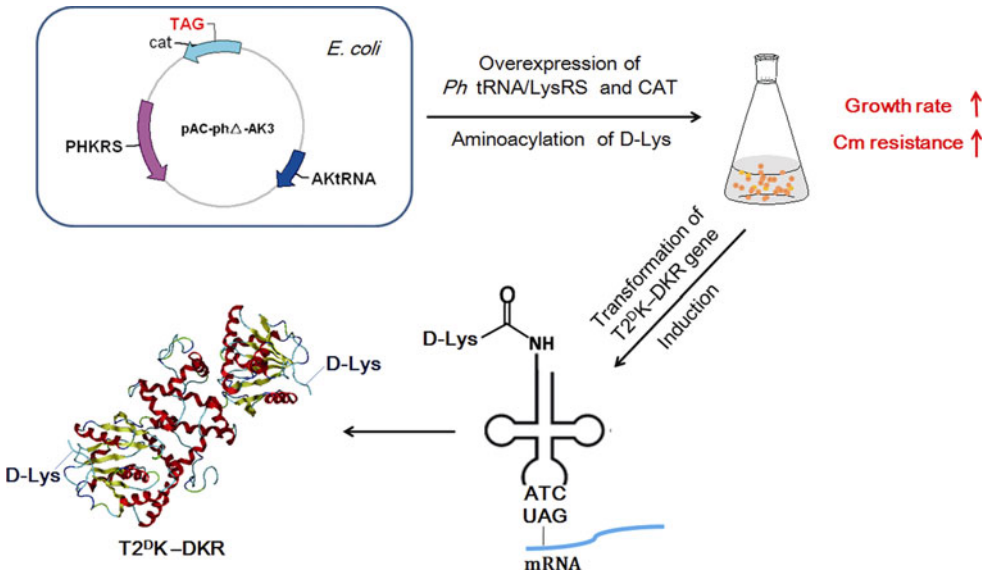

Fig. 1 Strategy for incorporation of D-Lys into diketoreductase by Ph tRNA/LysRS pair in *E. coli* cells

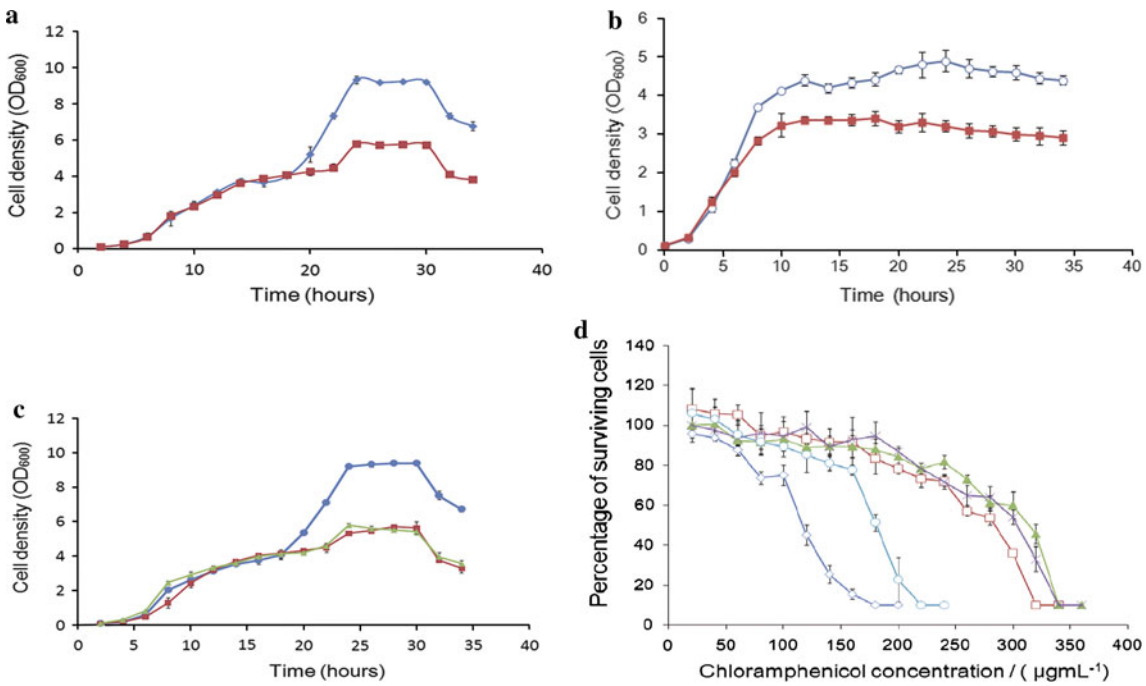

Fig. 2 Effects of D-Lysine on the growth and Cm resistance of *E. coli* cells harboring pAC-phΔ-AK3 plasmid. **a** Growth curves of *E. coli* BL21 (DE3) harboring pAC-phΔ-AK3 in the absence and presence of D-Lys. *Open squares* M9 medium, *open diamonds* M9 medium plus 5 mM D-Lys. **b** Growth curves of *E. coli* BL21 (DE3) cells in the absence and presence of D-Lys. *Open circles* M9 medium. *Open squares* 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-phΔ-AK3 plasmid. *Open circles* M9 medium plus 3.6 mM D-Lys. *Open squares* M9 medium plus 2.4 mM D-Lys. *Open triangles* M9 medium plus 1.2 mM D-Lys. *Error bars* represent standard deviations ($n = 3$). **d** Effects of various concentrations of D-Lys on Cm resistance of *E. coli* cells harboring pAC-phΔ-AK3 plasmid. *Open diamonds* M9 medium ($IC_{50} = 120$ μg ml^{-1}), *open circles* M9 medium plus 1.2 mM D-Lys ($IC_{50} = 180$ μg ml^{-1}), *open squares* M9 medium plus 2.4 mM D-Lys ($IC_{50} = 280$ μg ml^{-1}), multisymbol M9 medium plus 5 mM D-Lys ($IC_{50} = 320$ μg ml^{-1}), *open triangles* M9 medium plus 3.6 mM D-Lys ($IC_{50} = 340$ μg ml^{-1}). *Error bars* represent standard deviations ($n = 3$)

resistance against Cm if D-Lys could be specifically utilized by *Ph* LysRS to aminoacylate with *Ph* $tRNA^{Lys}_{CUS}$. A chloramphenicol (Cm) reporting system (Supplementary Fig. 2a) was introduced to *E. coli* cells, in which the 112th codon of CAT was replaced by an amber codon (Xie and Schultz 2005). Consequently, the effects of various concentrations of D-Lys on Cm resistance with *E. coli* cells harboring pAC-phΔ-AK3 were examined. Indeed, all

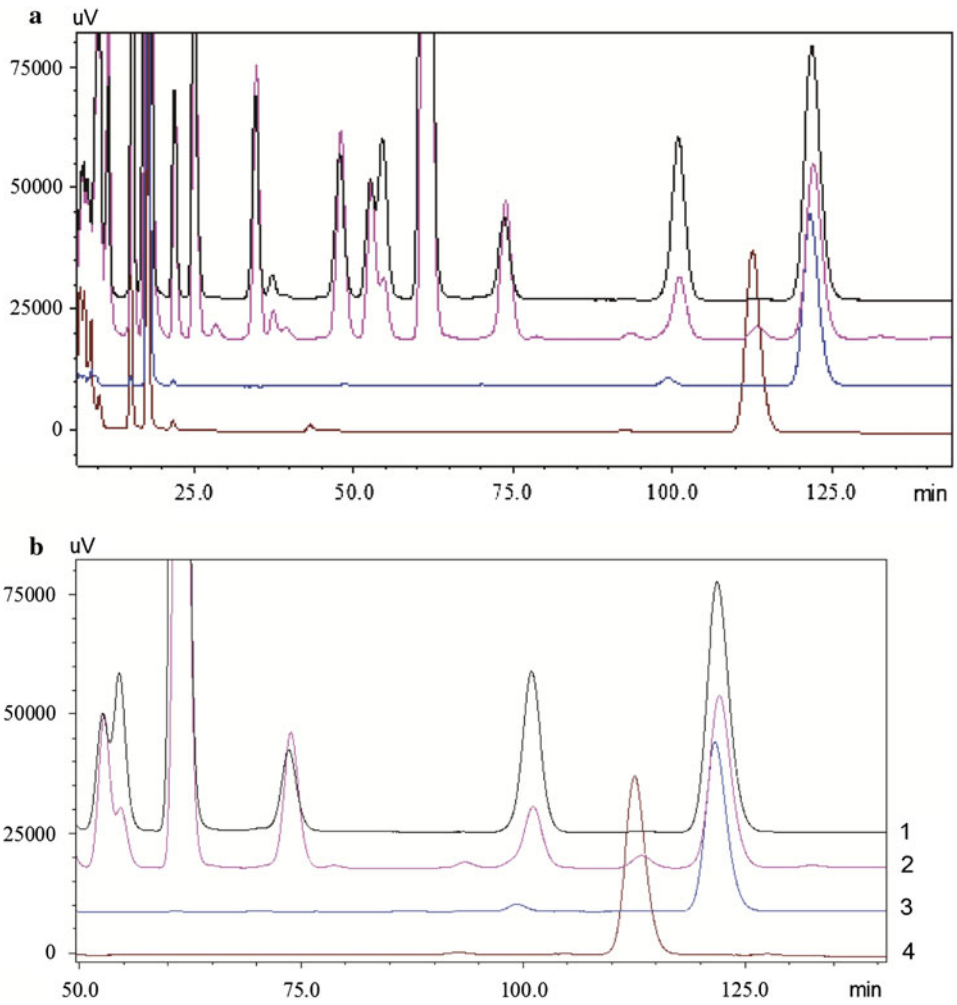

Fig. 3 Chrial HPLC analysis of the derivatives of hydrolyzates and Lysine stereoisomers. **a** Full Chiral HPLC chromatogram of the derivatives of hydrolyzate and lysine stereoisomers. **b** Expended region of the chiral HPLC chromatograms. *Line 1* Wild-type DKR; *Line 2* T2^{D}K mutant; *Line 3* authentic L-Lys; *Line 4* authentic D-Lys. The retention times of D-Lys and L-Lys derivatives were 113.3 and 122.2 min, respectively

tested concentrations of D-Lys significantly enhanced Cm resistance as shown in Fig. 2d. In the range of 1.2–3.6 mM D-Lys, the increase of Cm resistance was in a dose-dependent manner. These findings strongly supported that *Ph* $tRNA^{Lys}_{CUS}$/LysRS pair could utilize D-Lys in the culture medium.

Genetic incorporated D-lysine into diketoreductase

To further examine whether D-Lys can be incorporated into a foreign protein in the cells, we chose homodimeric DKR that has been extensively studied in our laboratory as a target protein (Chen et al. 2012; Wu et al. 2008, 2009, 2010, 2011a, b; Lu et al. 2010). Because the Thr residue at the second position of DKR is located in the N-terminus (Huang et al. 2012) and any substitutions with L-amino acids at this position do not influence enzyme activity (data not shown), this Thr residue was selected for switching to D-Lys. Thus, a pETduet-T2^{D}K-*dkr* construct was generated (Supplementary Fig. 2b), in which the 2nd codon of DKR gene was mutated from ACC to TAG. After co-transforming pETduet-T2^{D}K-*dkr* and pAC-phΔ-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^{D}K-DKR, was expressed by IPTG induction. Meanwhile, the plasmid carrying wild-type *dkr* gene (pETduet-*dkr*) was co-transformed with the same orthogonal pair and expressed under the same conditions. As expected from the suppression of tRNA, the expression level of T2^{D}K-DKR mutant was markedly lower compared with wild-type (Supplementary Fig. 3). The expressed T2^{D}K-DKR was purified by two-step chromatography to homogeneity (Supplementary Fig. 4). Measurements of enzyme activity indicated that the activity of T2^{D}K-DKR mutant was decreased compared with the wild-type (Supplementary Table 2), which might be due to the topological change by D-Lys at 2nd position to affect the cofactor binding site and consequently reduce the binding affinity between the cofactor and enzyme (Huang et al. 2012). Then, purified T2^{D}K-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^{D}K-DKR hydrolyzate at 113.3 min was clearly detected and quantified as in Fig. 3. After the same procedures of purification, hydrolysis and derivatization, wild-type DKR did not produce the peak from the derivatization of D-Lys. Because T2^{D}K-DKR contains 20 Lys residues and theoretically the ratio of D- versus L-stereoisomer should be 5.26 % if a D-Lys was completely incorporated into the protein, our experimental ratio was 5.87 ± 0.18 % ($n = 3$) after quantifying the peak areas in the HPLC chromatograms. This incorporation ratio confirmed that only one D-Lys was selectively incorporated into T2^{D}K-DKR protein, and the extra amount of D-Lys could be from the racemerization of L-Lys during the acid hydrolysis.

To further prove the incorporation of D-Lys at a specific site, mass analyses were conducted for the peptide fragments after trypsin digestion. As shown in supplementary Fig. 5, the mass spectrum of trypsin digested T2^{D}K showed a major peak (*m/z* 2, 616.3 Da), corresponding to the peptide fragment containing D-Lys (GSSHHHHHH SQDPM^{D}KG ITNVTVLG, theoretical mass 2, 614.9 Da), whereas digestion of wild-type DKR produced the fragment with Thr at this position (Supplementary Fig. 5; Supplementary Table 3). The mass data strongly confirmed the site-specific incorporation of D-Lys into DKR.

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$^{Lys}_{CUS}$/LysRS pair towards D-Lys, which is in an agreement with a previous report on the substrate specificity (Wang et al. 2007).

Conclusion

We have demonstrated that the expression of *Ph* tRNA$^{Lys}_{CUS}$/LysRS pair could reduce the toxicity of D-Lys to *E. coli* cells. Moreover, increase of Cm resistance also suggested *Ph* LysRS could be highly stereoselective towards D-Lys in vivo when excess amount of D-Lys is present in the culture medium. After expressing T2^{D}K-DKR mutant, chiral HPLC analysis of D-Lys derivatives and mass spectrometry of peptide fragments 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.

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