Molecular engineering of a PheS counterselection marker for improved operating efficiency in *Escherichia coli*

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Escherichia coli phenylalanyl-tRNA synthetase, α-subunit (ePheS) can be useful as a counterselection marker since its A294G variant misincorporates 4-chloro-phenylalanine (4CP) into cellular proteins during translation, thereby causing cell death. The drawback of this method is that selection must be performed in minimal or semisynthetic medium to avoid interference from phenylalanine in the medium. Here, I reengineered ePheS for improved 4CP incorporation efficiency, obtaining variants (T251A/A294G and T251S/A294G) that exhibited high lethality in Luria-Bertani medium (LB) containing 4CP. These new variants were superior to the A294G variant when used as a counterselection marker in vector curing experiments.

Escherichia coli is the most widely used host microorganism for genetic engineering experiments. In recent decades, numerous vectors containing various selection markers (e.g., antibiotic resistance genes) have been developed. Cells that gain vectors are readily selected by exploiting such positive selection markers. However, current methods to select clones that lose vectors are rather poor due to the lack of negative (or counter-) selection markers available, even though they are frequently used in genome recombineering, genome random mutagenesis (1), and plasmid curing experiments (2).

Phenylalanyl-tRNA synthetase catalyzes the charging of $tRNA^{Phe}$ with phenylalanine. Because of its central role in life, the substrate specificity of the enzyme is extremely high. Erroneous aminoacylation with noncognate amino acids causes cell death (3). In *E. coli*, the enzyme forms an $(\alpha\beta)_2$ heterotetramer (4) in which the α subunit is named PheS. The A294G mutant of PheS ($ePheS^{A294G}$) is known to misincorporate 4-chloro-phenylalanine (4CP) into proteins both in vitro (4,5) and in vivo (6,7). Exploiting this lethal effect, $ePheS^{A294G}$ was used as a positive selection marker; only recombinant vectors carrying an insert within the $ePheS^{A294G}$ gene can grow in the presence of 4CP (8). The merit of using $ePheS^{A294G}$ is that it shows a dominant phenotype over the wild-type chromosomal allele; thus, no specially designed strain (i.e., deletion mutant) is necessary. The drawback, however, is that selection must be achieved in minimal (6) or semisynthetic yeast extract glucose (YEG) (8) media to avoid interference from phenylalanine in the medium (9). Thus, I engineered ePheS for higher lethality for use in common bacterial growth media.

I first targeted Ala294 by saturation mutagenesis (10,11) because a previous study focused only on several mutants (Ser, Thr, Gly, Val, and Cys) (6). The ePheS gene was cloned into a high expression vector, pJExpress404, to yield pJePS1 (Supplementary Table S1). Degenerate primers (Supplementary Table S2) were used to randomize Ala294 to create a library, from which 96 colonies were randomly picked and screened for viability in YEG/ampicillin (Amp), 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and 10 mM 4CP. The clone showing the lowest OD_{600} value had a Gly294 (GGG codon) (Supplementary Figure S1).

Next, Thr251, which is involved in substrate recognition (4), was targeted for mutagenesis. Saturation mutagenesis was applied using $ePheS^{A294G}$ as a parent. In total, 96 clones were screened for viability in Luria-Bertani medium (LB)/Amp, 1 mM IPTG, and 10 mM 4CP, which yielded more than 20 clones showing no growth. Thus, I reduced the concen-

METHOD SUMMARY

The *Escherichia coli* phenylalanyl-tRNA synthetase, α-subunit gene was engineered for improved incorporation efficiency of a toxic substrate analogue, 4-chloro-phenylalanine (4CP). The gene was efficiently used as a counterselection marker in *E. coli* in common bacterial growth media such as LB that contained 4CP.

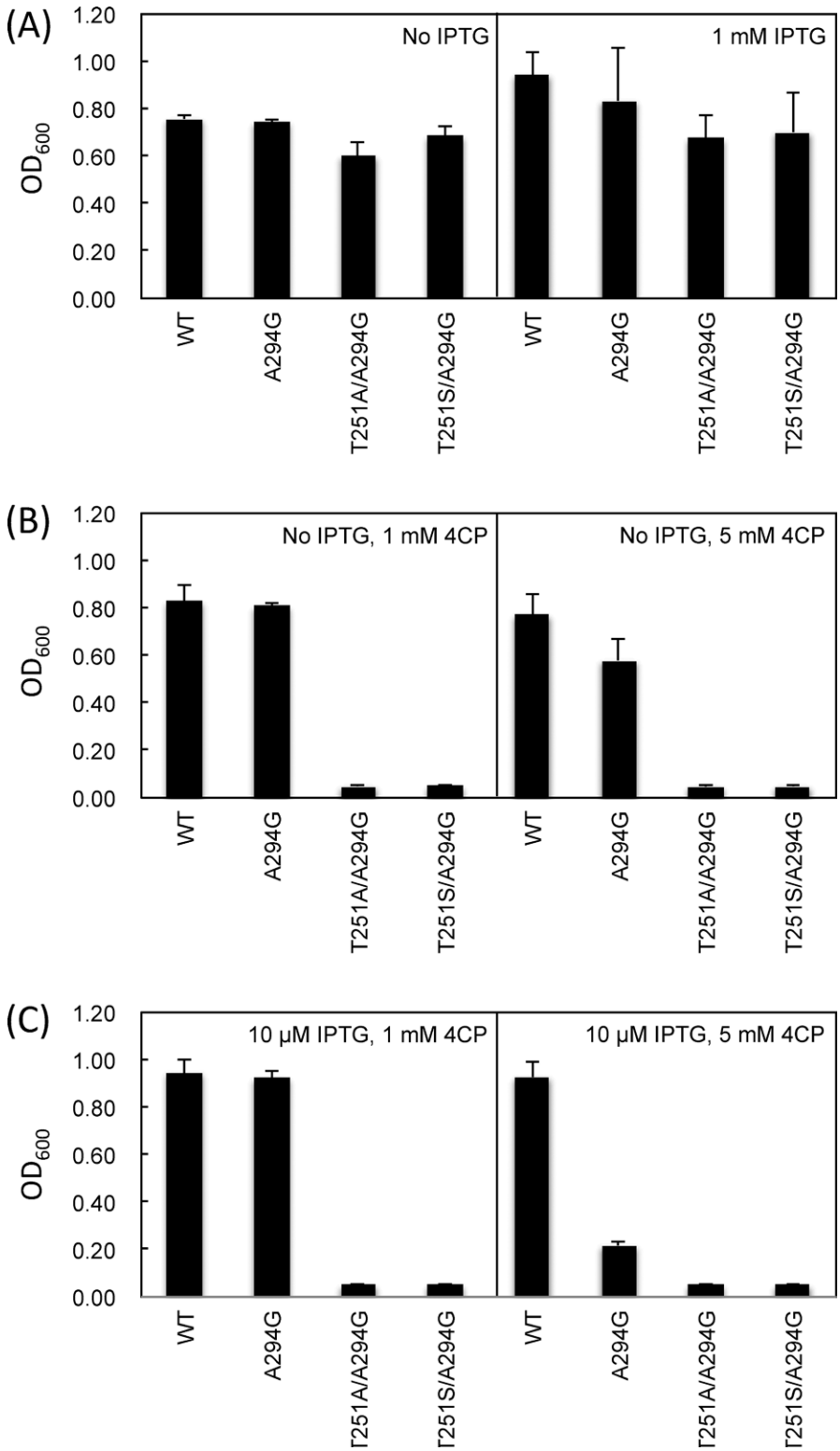

Figure 1. Growth of *Escherichia coli* JM109 carrying pJePS1 expressing wild-type and variant *E. coli* phenylalanyl-tRNA synthetase, α-subunit (ePheS) genes. (A) Protein toxicity test. Cells were grown in 1 mL of LB/Amp containing 0 or 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) in a 96-well plate with shaking (1200 rpm) at 37°C for 16 h. (B and C) Susceptibility test. Cells were grown in LB/Amp containing (B) 0 mM IPTG and 1 or 5 mM 4-chloro-phenylalanine (4CP) or (C) 10 μM IPTG and 1 or 5 mM 4CP in a 96-well plate with shaking (1200 rpm) at 37°C for 16 h. In both cases, 170 μL of the cells were transferred to a 96-well plate, and the OD_{600} was measured. Values: mean ± SD; $N = 8$.

trations of IPTG (to 0 and 10 μM) and 4CP (to 1 mM); 10 clones showed no growth even at 0 mM IPTG, when only leaky expression should occur. DNA sequencing revealed the presence of Ala or Ser (five clones each). Note the codon variation in these variants, confirming the reliability of the screen (Supplementary Table S3). Clones B2 (Ala251) and E4 (Ser251) were used for further study.

When grown in LB/Amp (no 4CP), all of the clones showed similar OD_{600} values regardless of the presence or absence of IPTG, indicating a lack of protein toxicity (Figure 1A). This is advantageous because SacB (levansucrase) of *Bacillus subtilis* (12), the most commonly used counterselection marker in *E. coli*, suffers from a protein toxicity problem that causes false positives (13). As for susceptibility to 4CP (Figure 1, B and C), when the cells were grown in LB/Amp with 4CP, even in the absence of IPTG, specific growth inhibition was observed for the double mutants but not for wild-type and $ePheS^{A294G}$.

I next cloned the $ePheS^{T251A/A294G}$ gene into pUC18K [containing a kanamycin (Km) resistance (Km^R) gene] and pUC18Z [containing a zeomycin (Zeo) resistance (Zeo^R) gene] downstream of the antibiotic resistance gene in each vector to yield pUC18K_ePAG2 and pUC18Z_ePAG2, respectively (Supplementary Figure S2). JM109 cells were transformed with these vectors and grown on LB/Km and LB/Zeo agar plates containing various concentrations of 4CP. No colonies appeared on plates containing 1.25 mM 4CP, but more than 10^7 colonies appeared in its absence, demonstrating high counterselection efficiency (Supplementary Table S4). Counterselection was less efficient when the $ePheS^{A294G}$ gene was used; a higher concentration of 4CP was required for selection.

Finally, I applied the $ePheS^{T251A/A294G}$ marker to strain improvement. The mCherry fluorescence protein gene was cloned into pUC18K_ePAG2 (expression plasmid designated pUC18K_ePAG2_mCN1), which was introduced into *E. coli* JM109. Fluorescent colonies that appeared on LB/Km/IPTG plates were streaked on LB/4CP plates to isolate cured

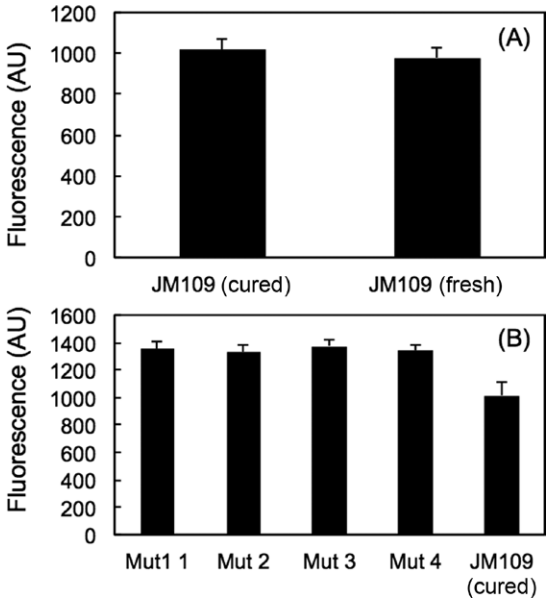

Figure 2. mCherry fluorescence of wild-type and mutant *Escherichia coli* JM109. (A) JM109 (cured) denotes the strain that was transformed with pUC18K_ePAG2_mCN1 and cured on an LB/4-chloro-phenylalanine (4CP) plate. JM109 (fresh) denotes the strain without transformation/curing. Cells were transformed with pUC18K_ePAG2_mCN1, and the fluorescence intensity was measured. (B) Comparison of fluorescence intensity between the mutants and JM109. In both cases, cells were grown in 1 mL of LB/Km containing 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) in a 96-well plate with shaking (1200 rpm) at 37°C. After 12 h, 170 µL of the cells were transferred to a 96-well plate, and the fluorescence intensity (excitation, 509 nm; emission, 530 nm) was measured. Values: mean ± SD; $N = 4$.

hosts. Loss of the vector was verified by the disappearance of fluorescence and sensitivity to Km. Cured JM109 and fresh JM109 were then retransformed with the mCherry expression vector. In LB/Km/IPTG medium, both strains showed equivalent fluorescence, confirming successful vector curing (counterselection) and retransformation (Figure 2A).

pUC18K_ePAG2_mCN1 was next introduced into a mutant library (created by UV irradiation). Approximately 2000 mutants were screened on LB/Km/IPTG plates, and four clones showing enhanced fluorescence were identified. They were streaked on LB/4CP plates to isolate cured hosts. The cured mutants and wild-type JM109 were retransformed with the mCherry expression vector. Fluorescence analysis indicated that all strains showed enhanced (×1.3) mCherry expression (Figure 2B).

I next replaced the mCherry gene with the green fluorescence protein (GFP) gene (expression plasmid designated pUC18K_ePAG2_GFP). Three types of GFP genes (GFP^{Bsu}, GFP^{Eco}, and GFP^{Sco}) that have different synonymous mutations were used (14). All mutants showed enhanced (×1.3–1.9) fluorescence (Supplementary Figure S3), suggesting the potential utility of the mutants as an expression host.

In conclusion, the ePheS variants (T251A/A294G and T251S/A294G) described in this study can be advantageously used as a counterselection marker with high selection efficiency. Effective selection was confirmed in LB, YT, and 2xYT media using *E. coli* strains JM109, DH5α, NEB turbo, and BL21(DE3). PheS functions in a complex with PheT (β-subunit); thus, the present marker can be used in *E. coli*. However, because some enterobacterial (e.g., *Shigella*, *Salmonella*, and *Citrobacter*) PheS enzymes are >98% identical in sequence, the marker described should also be usable in these bacteria.

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Competing interests

The author declares no competing interests.

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