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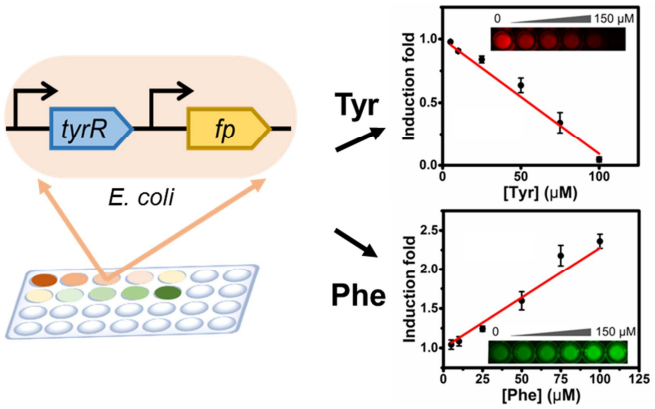

Transcription Factor-Based Biosensor for Detection of Phenylalanine and Tyrosine in Urine for Diagnosis of Phenylketonuria

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ABSTRACT

Clinicians require a simple quantitative method for the detection of both phenylalanine and tyrosine to facilitate the diagnosis of phenylketonuria, a common inherited disorder of amino acid metabolism. In this study, we developed a novel whole-cell biosensor for the quantification of phenylalanine and tyrosine through the expression of red and green fluorescent proteins. The proposed system responds specifically and sensitively to phenylalanine/tyrosine without interferences from other amino acids. Furthermore, the precision of the biosensor was evaluated using specimens of normal human urine by LC-MS.

KEYWORDS: phenylalanine, tyrosine, *E. coli*, *tyrR*, phenylketonuria.

Introduction

Phenylalanine (Phe) is an important marker for the diagnosis of phenylketonuria (commonly known as PKU). PKU is an inherited disorder of amino acid metabolism, which can lead to increased Phe levels resulting from a deficiency of phenylalanine hydroxylase (PAH).[1-7] PAH plays an important role in the hydroxylation of Phe into tyrosine (Tyr). Many patients with this disorder develop profound irreversible neurological damage through the accumulation of Phe and phenylpyruvic acid (PPA). The conventional approach to treating this disorder involves dietary control over foods rich in Phe. In addition, an elevated Phe/Tyr ratio is commonly observed among PKU patients (a ratio above 3; typically less than 1).[8] This suggests that the continuous measurement and quantification of both Phe and Tyr may be helpful in maintaining stringent control over the dietary intake of Phe, thereby preventing the emotional instability and/or the development of physical disorders.[9, 10] Moreover, Phe/Tyr ratios have been used to confirm the diagnosis of PKU and hyperphenylalaninemia effectively.[11, 12]

Numerous methods have been developed for the detection of Phe, including enzymatic commercial assay kits, tandem mass spectrometry, and high-pressure liquid chromatography.[13-18] These methods provide high sensitivity and accuracy; however, pre-column derivatization is laborious and time consuming and the testing

apparatus/reagents used for real-time monitoring are expensive. Kim et. al previously developed a cell-based *E. coli* auxotroph to enable the detection of Phe with a high degree of sensitivity.[19] Unfortunately, none of the existing methods enable the measurement of Phe and Tyr concentrations simultaneously. In this study, we developed a whole-cell biosensor for the simultaneous detection of Phe and Tyr based on the transcriptional regulation of TyrR in *E. coli*.[19-22]

The biosynthesis of Phe and Tyr in *E. coli* are controlled by the DNA binding protein TyrR, the dimer of which regulates the activation of *tyrP* and *mtr* in the presence of Phe. However, in the presence of Tyr and ATP, TyrR dimers self-associate to form hexamers, which regulate the repression of *aroF, tyrP, aroP* and *tyrB*. TyrR protein binds to the consensus DNA sequence $TGTAAAN_6TTTACA$, (referred to as TyrR boxes), which are classified as strong or weak. In the presence of Phe, the *in vitro* binding of strong boxes by TyrR dimers strongly induces the expression of genes by facilitating the binding of RNA polymerase to the promoter, or by facilitating the binding of a second dimer to an adjacent weak box. Weak boxes are less strongly associated with the consensus sequence, and are bound by the TyrR hexamer. This results in repressed gene expression due to the compromised ability of bound RNA polymerase to form open complexes or leave the promoter.[23-26] We therefore designed the biosensor by screening various promoters and examining their activities

in the presence of Phe and Tyr.

2. Experimental methods

2.1 Bacterial strains and growth conditions

E. coli DH5α cells were used as the hosts for molecular cloning. They were cultivated in LB broth under shaking at 250 rpm at 37°C. The strains, plasmids, primers, and construct details are provided as Supporting Information and listed in Tables S1, S2, and S3, respectively.

2.2 Measurements of fluorescence intensity

Biosensor strains were grown in LB broth at 37°C overnight. The cells were diluted (1:50; v%) in M9 medium (supplemented with 0.4% glucose, 0.24 mg/mL $MgSO_4$, 11.1 μg/mL $CaCl_2$, 0.3 μM thiamine hydrochloride, and 50 μg/mL kanamycin) with Phe/Tyr and incubated at 37°C for 24 hours. The IPTG-induced cells (PlacUV5) were diluted in M9 medium under the same conditions until reaching an OD_{600} of approximately 0.4, whereupon they were induced using 100 μM of IPTG (Amresco). The OD_{600} values and fluorescence intensity were measured (from 24 h post-induction) using a Synergy HT microplate reader (BioTek, Winooski, VT) equipped with an excitation/emission filter (530 nm/590 nm for RFP, 485 nm/528 nm for GFP). Fluorescence images were obtained using a fluorescence image analyzer (GE Amersham Imager 600). All experiments were conducted in triplicate.

The amino acids examined in this study were as follows: phenylalanine (Acros), tyrosine (Merck), phenylpyruvic acid (Sigma-Aldrich), phenylacetic acid (Sigma-Aldrich), tryptophan (Sigma-Aldrich), alanine (Acros), arginine (Sigma-Aldrich), asparagine (Sigma-Aldrich), aspartic acid (Sigma-Aldrich), cysteine (Sigma-Aldrich), glutamic acid (Acros), glutamine (Sigma-Aldrich), glycine (Amresco), histidine (Acros), isoleucine (Sigma-Aldrich), leucine (KYOWA), methionine (Sigma-Aldrich), proline (Acros), serine (Girasol Bio), threonine (TCI), and valine (Aldrich).

2.3 Liquid chromatography-mass spectrometry (LC-MS) measurements:

Measurements were performed using an ACQUITY Ultra Performance Liquid Chromatography (Waters, Manchester, UK) in line with an API 3000 Quadrupole mass spectrometer (SCIEX, Concord, ON, Canada). Separations were achieved using a C18 Hypersil GOLD column (50 mm × 2.1 mm, 1.9 μm) with isocratic elution (mobile phase A: 10 mM ammonium acetate solution in water; and mobile phase B: 10 mM ammonium acetate solution in water/acetonitrile (10:90 v/v). Mobile phases A and B were both adjusted to pH 5.5 using acetic acid. The elution program was as follows: 10% B held for 5 min; mobile phase flow rate of 0.4 mL/min; of electrospray potential of +5 kV (ESI positive mode).

2.4 Urine sample preparations

Urine samples were collected from healthy volunteers and stored at -20°C prior to use (the study was approved by the institutional review board of Antai Medical Care Cooperation Antai Tian-Sheng Memorial Hospital). The urine samples were diluted using 2xM9 medium under the conditions described above for cell-based assays. Aliquots (1.0 mL) of urine were centrifuged at 12,000 × g for 10 min. The supernatant of the urine (5 μL) was mixed with a methanol/water solution (50:50 v/v, 195 μL) and vortexed for 20 second. A sample of the supernatant (20 μL) was then injected into the LC-MS system.

3. Experimental results and discussion

3.1 Construction and characterization of biosensor for the detection of Phe and Tyr

We began by selecting five promoters (*aroF, tyrP, aroP, mtr*, and *aroG*) for use in examining the expression of fluorescent protein(s) in the presence of Phe and Tyr, respectively. TyrR was driven by a constitutive promoter (P14) on the same plasmid. Fig. 1A presents a schematic diagram of the proposed biosensor. Cells were grown with Phe or Tyr in M9 minimal medium supplemented with 0.4% glucose at 1 mM for 24 h. We recorded the end-point induction folds (induced relative to uninduced fluorescence intensities/OD) of the five strains (Fig. 1B-F). In the presence of Tyr, strains carrying *ParoF-fp* and *ParoP-fp* respectively presented 22-fold and 4-fold decreases in intensity values, whereas *Pmtr-fp* produced a 5-fold increase in intensity

values. The fluorescence signals of PtyrP and ParoG did not vary. In the presence of Phe, strains driven by PtyrP and Pmtr respectively presented 2.5-times and 4.2-times increases in fluorescence intensity, whereas ParoP presented a 2.5-fold decrease. The signals of ParoF and ParoG remain unchanged. Among the five promoters, Pmtr presented the strongest activation of Phe, whereas ParoF presented the strongest repression of Tyr. The above values are summarized in Table S4.

Regulatory proteins generally interfere with the performance of sensor strains. Specifically, TyrR protein levels have been shown to have a dramatic impact on the levels of various forms of the TyrR protein.[23, 24] We therefore investigated the correlation between the responsive fluorescence signals and the concentrations of TyrR, by comparing endogenous expression with one inducible promoter (PlacUV5) and two synthetic constitutive promoters (P14 and P14-1).[27, 28] P14-1 (a weak derivative of P14) was generated by introducing random mutations in two nucleotides within the −10 box. Fig. S1 illustrates the red fluorescence intensities induced by the three promoters on the pBbE5k plasmid. P14 presented the strongest promoter activity, followed by PlacUV5. It is interesting to note that increasing the number of copies of TyrR had only a minor effect on ParoP and ParoG. Nonetheless, the fluorescence intensity of Pmtr increased with the concentrations of the regulatory proteins. ParoF and PtyrP also presented indications of TyrR-dependent regulation. To enable the

simultaneous reporting of two distinct substrates, we selected promoters that are Tyr-sensitive and Phe-insensitive, and vice-versa. Thus, two strains carrying P14-TyrR with *ParoF-rfp* or *PtyrP-gfp* were used in all subsequent tests.

We then sought to determine whether a dose-response relationship exists between the concentration of Phe/Tyr and the induction folds of strains carrying *ParoF-rfp*. As shown in Fig. 2A, the fluorescence intensity dropped to 60% when the concentration of Tyr was increased to 50 μM. As shown in the photograph (in Fig. 2B inset), RFP can only be observed under lower Tyr concentrations. The fluorescence response presented a high degree of linearity (R^2=0.977) with the concentration of Tyr, ranging from 5-100 μM (Fig. 2B). The limit of detection (LOD) for Tyr (based on 3б/slope) was calculated to be 4.9 μM. It should be noted that the average induction folds remained qualitatively unchanged when the Phe concentration was adjusted from 0-1000 μM. We also investigated the dose-response relationship between Phe/Tyr and induction folds in the strain carrying *PtyrP-gfp*. Unlike RFP, we observed an increase in GFP following an increase in the concentration of Phe from 0-1000 μM, (Fig. 2C). Furthermore, the induction folds presented a linear correlation with the concentration of Phe (R^2=0.996, in a concentration range of 5-100 μM) and the LOD was 3.7 μM, (Fig. 2D). GFP induction was not observed in the presence of Tyr at concentrations of up to 1000 μM. The inset of Fig. 2D presents a green fluorescence image of a 96-well

plate containing samples with various concentrations of Phe.

3.2. Selective responses of proposed biosensor to Phe and Tyr

It is common to observe in the urine of patients with PKU an increase in the quantity of PPA and phenylacetic acid (PA), which are catabolic byproducts of Phe metabolism. [29] Thus, we evaluated the selectivity of the proposed sensor by examining the fluorescence induction for twenty amino acids, PPA, and PA at 100 μM. We examined the fluorescence intensity of GFP and RFP in the presence of various substrates of strains carrying *PtyrP-gfp* and *ParoF-rfp*, respectively. We did not observe the induction of RFP or GFP when the substrates were added to the medium; however we did observe the induction of PPA in cells carrying *PtyrP-gfp*. Notably, the induction of PPA was similar to that of Phe (Fig. 3). We therefore examined the increase in GFP in the presence of PPA at various concentrations. As shown in Fig. S2, there was a significant increase in GFP intensity when the concentration of PPA was increase beyond 100 μM. PPA levels in the blood or urine of healthy individuals are normally very low.[30, 31] Thus, there should be negligible interference from PPA in biological samples. We also tested for interference between Phe, Tyr, and PPA. Our results revealed that when the three substrates coexist, the induction folds of sensor cells were nearly identical to those containing Phe or Tyr alone (Fig. S3).

We investigated the feasibility of integrating *tyrR*-inducible PtyrP and ParoF in the same strain by constructing two strains harboring various combinations of the two-plasmid systems including *P14-tyrR-PtyrP-gfp*::pBbE5k/*ParoF-rfp*::pKT230, and *P14-tyrR-ParoF-rfp*::pBbE5k/*PtyrP-gfp*::pKT230, respectively. It is interesting to note that the two-plasmid system did not present a Phe/Tyr dependent dose-response relationship (Fig. 4A and B). Similar results were observed when PtyrP was substituted for ParoF. These results may be attributed to the fact that the constitutive promoter used to drive *tyrR* regulatory proteins in our sensor system lacked the strength required for the expression/repression of GFP/RFP. There was a significant reduction/increase in the expression of *PtyrP-gfp* in the presence of Phe/Tyr (Fig. 4A). This is consistent with the results in Fig. 1C (P14 and P14-1). Moreover, ParoF was shown not to repress RFP in the presence of Tyr (Fig. 4B), which is consistent with the results in Fig. 1B (P14 and w/o tyrR). To circumvent this problem and avoid the complex regulation between *tyrR* and substrates, we employed a single-plasmid system to ensure that the GFP/RFP protein would be expressed in a Phe/Tyr-dependent manner.

3.3 Human urine analysis

We also evaluated the detection precision of the proposed biosensor when applied to the diagnosis of PKU from human urine specimens. Human urine samples were

diluted at a 1:1 ratio in 2X M9 for the cell-based quantification of Phe and Tyr. Each measurement was replicated three times for every sample. Table 1 lists the analytical results, including standard deviation, coefficient of variation, and ratio (compared with the values obtained using LC-MS). The accuracy (ranging from 120-137%) and precision CVs (3.8-17.2%) were all within an acceptable range.

4. Conclusions

In this study, we developed a novel approach to the analysis of Phe/Tyr in urine samples using cell-based biosensor technology. Table S5 compares the linear range and LOD of various cell-based biosensors as well as those using enzymatic and electrochemical reactions. The biosensor developed in this study presented a broad dynamic range with sensitivity equivalent to or exceeding that of existing sensors in the detection of Phe. The proposed biosensor also presented outstanding performance in the detection of Tyr, which is rarely addressed in the literature. The urine sample has a higher concentration of Phe than blood (the clinical range of Phe in human urine is 20–60 mM for people with PKU).[32] Thus, the proposed method could provide potential applications and simple solutions to the analysis of Phe/Tyr content in urine of PKU patients. Future studies could focus on genetic engineering of the promoter regions to enhance the selective recognition of Phe/Tyr. This could yield performance models useful to the development of new applications for the *tyrR* regulon.

Acknowledgements

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Supporting Information

Supporting Information associated with this article can be found in the online version. Experimental details, RFP/GFP characterizations, detailed of LC-MS assays, and preparation of urine samples.

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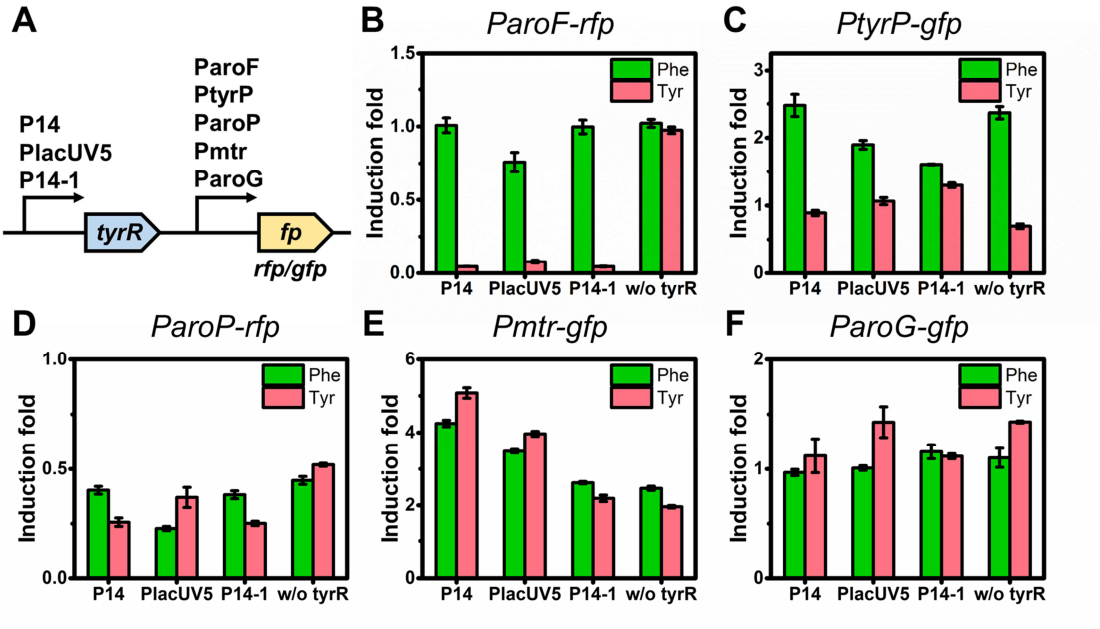


Fig. 1 (A) The genetic organization of proposed sensors. Plasmids contain combinations of *tyrR* (driven by P14, PlacUV5, P14-1, or basal level of tyrR) and TyrR-regulated promoters were constructed. (B)-(F) Induction folds of five promoters with various levels of TyrR in the presence of Phe or Tyr at 1 mM. w/o tyrR: the plasmid does not contain *tyrR* gene.

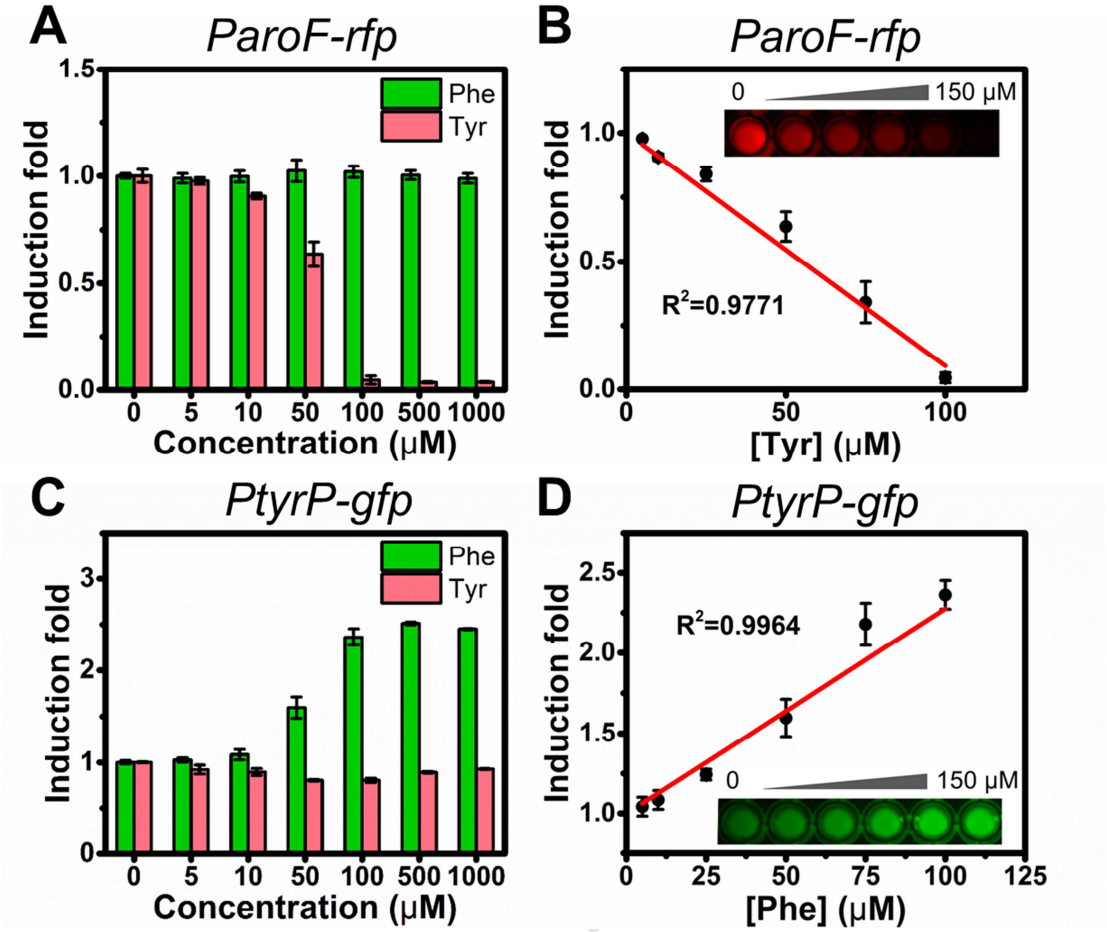


Fig. 2 Dose-response curves of (A) *ParoF-rfp* and (C) *PtyrP-gfp* in the presence of Tyr and Phe, respectively. Linear ranges for (B) Tyr and (D) Phe were both 5-100 μM, and LODs were 4.9 and 3.7 μM, respectively. Insets: the photograph of (B) RFP and (D) GFP in the presence of Tyr and Phe, respectively.

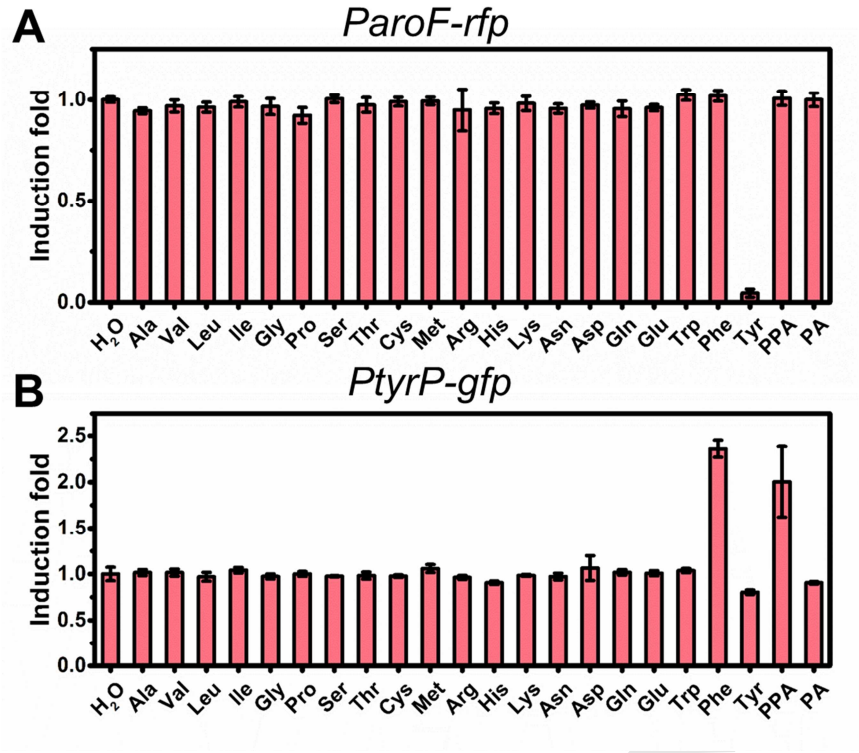


Fig. 3 The selectivity of the proposed sensor was examined. The fluorescence inductions of sensor cells carrying (A) *ParoF-rfp* and (B) *PtyrP-gfp* in the presence of twenty amino acids, PPA and PA at 100 μM were recorded.

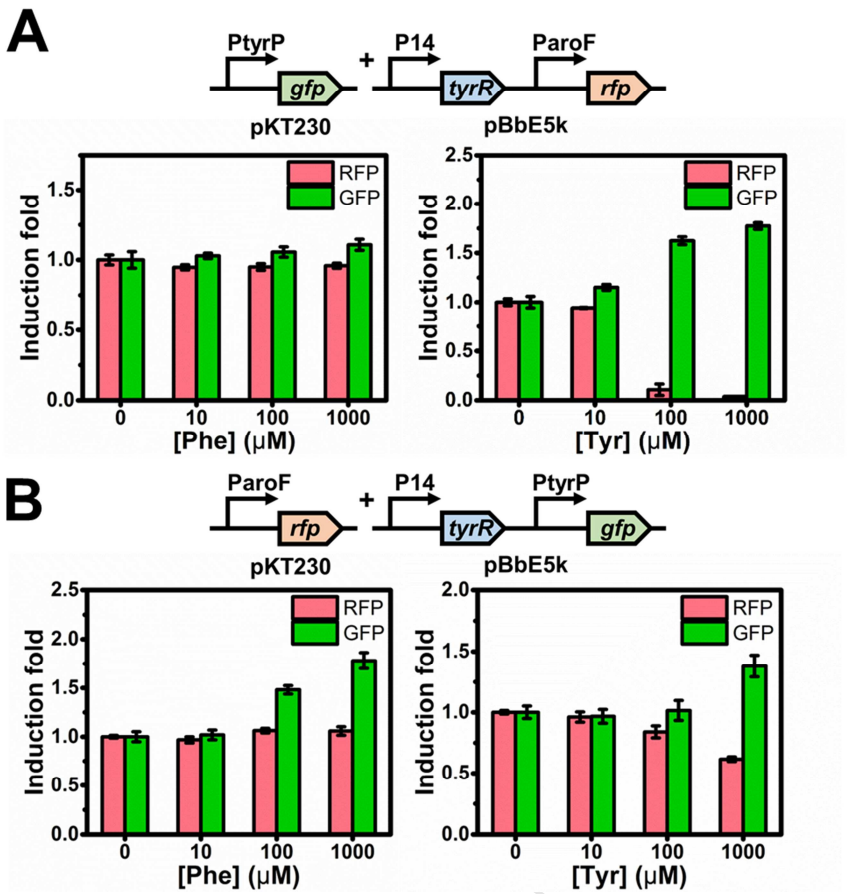
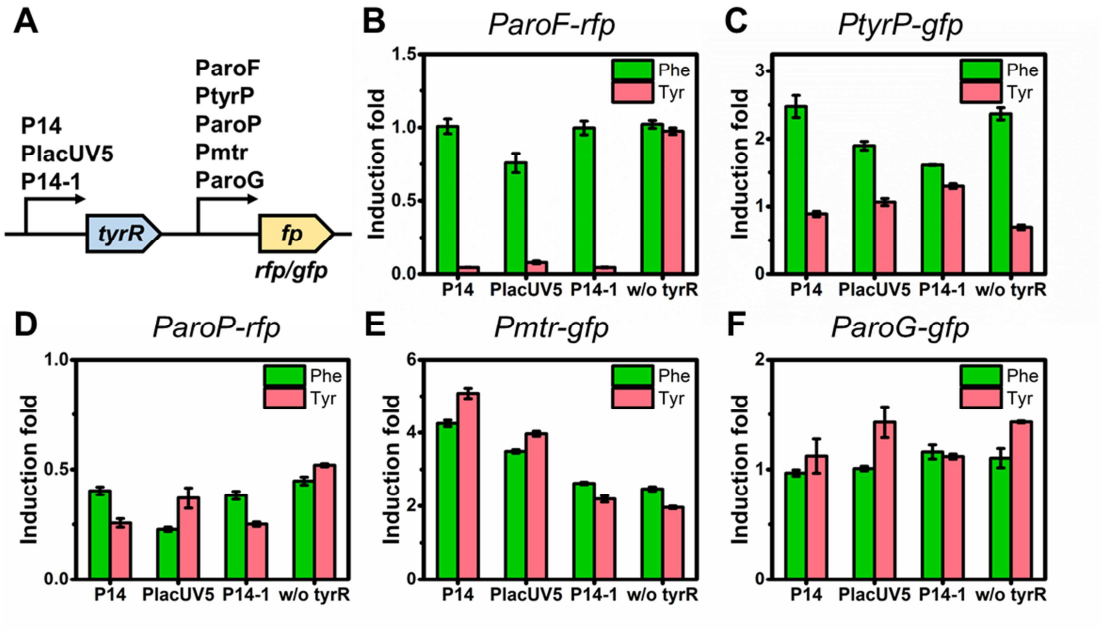
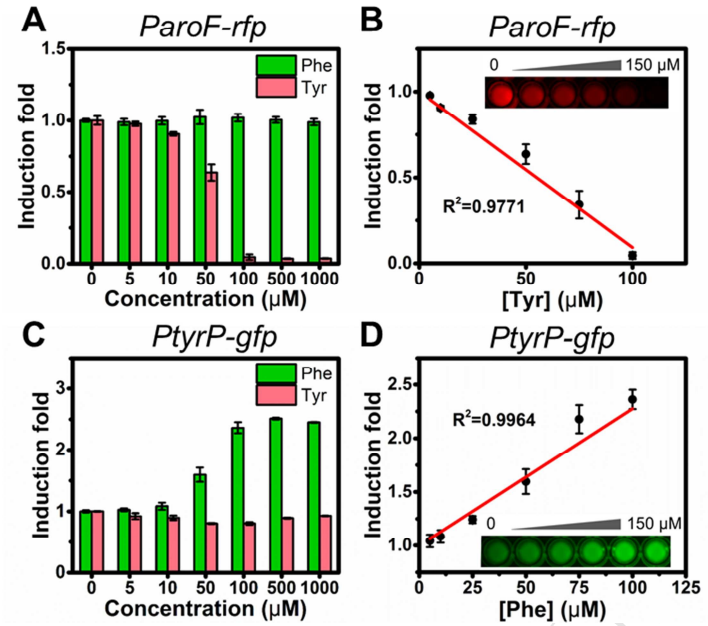


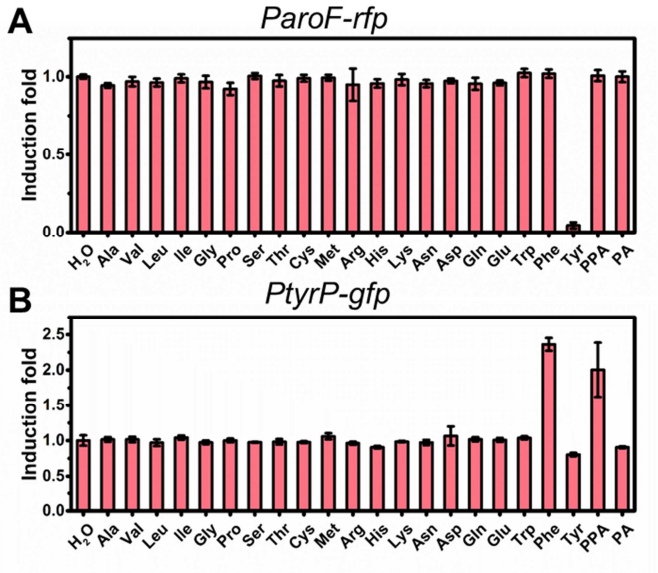
Fig. 4 The applicability of integrating *tyrR*-inducible PtyrP and ParoF in the same strain. The genetic organization and fluorescence inductions of cells containing (A) *PtyrP-gfp*::pKT230 and *P14-tyrR-ParoF-rfp*::pBbE5k (B) *ParoF-rfp*::pKT230 and *P14-tyrR-PtyrP-gfp*::pBbE5k in the presence of Phe and Tyr.

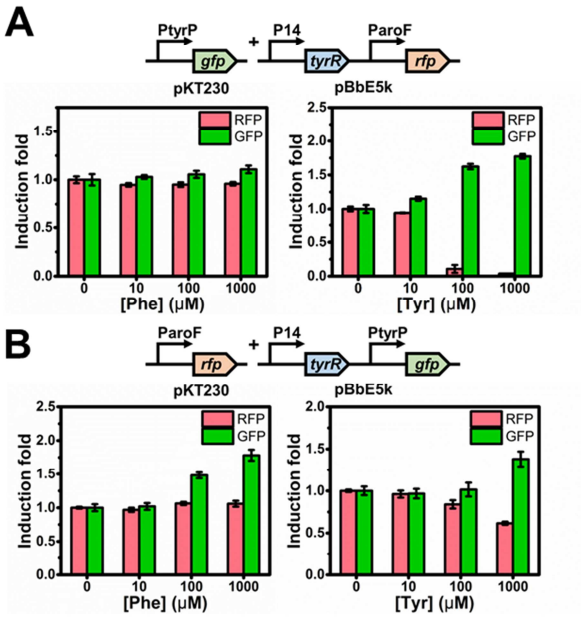
Table 1 Comparison of cell-based assay with LC-MS. SD, standard deviation. CV, coefficient of variation. Ratio: value of cell-based assay/LC-MS.

Sample	Amino acid	Mean (μM)	SD	CV (%)	Ratio (%)
1	Phe	51	1.9	3.8	124
	Tyr	28	1.9	6.9	120
2	Phe	85	7.22	8.5	137
	Tyr	70	9.17	13.1	123
3	Phe	49	8.4	17.2	126
	Tyr	24	2.4	9.8	129









- A novel cell-based approach to the analysis of phenylalanine and tyrosine in urine samples was developed.
- The whole-cell biosensors exhibited great sensitivity and selectively for the detection of phenylalanine and tyrosine.
- The accuracy and precision of proposed sensor for normal human urine samples were validated with LC-MS with satisfactory results.