Received: 13 September 2017 | Revised: 29 November 2017 | Accepted: 13 December 2017
DOI: 10.1002/bit.26521

ARTICLE

WILEY BIOTECHNOLOGY & BIOENGINEERING

# Engineering tunable biosensors for monitoring putrescine in *Escherichia coli*

Xue-Feng Chen[1] | Xiao-Xia Xia[1] | Sang Yup Lee[2] | Zhi-Gang Qian[1]

[1] State Key Laboratory of Microbial Metabolism, Joint International Research Laboratory of Metabolic & Developmental Sciences, School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai, People's Republic of China

[2] Metabolic and Biomolecular Engineering National Research Laboratory, Department of Chemical & Biomolecular Engineering (BK21 Program), BioProcess Engineering Research Center, Bioinformatics Research Center, and Institute for the BioCentury, KAIST, Yuseong-gu, Daejeon, Republic of Korea

**Correspondence**
Zhi-Gang Qian, State Key Laboratory of Microbial Metabolism, Joint International Research Laboratory of Metabolic & Developmental Sciences, School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, 800 Dong Chuan Road, Shanghai, 200240, People's Republic of China.
Email: zgqian@sjtu.edu.cn

**Funding information**
National Natural Science Foundation of China, Grant number: 21406138; The National Key Research and Development Program of China, Grant number: 2016YFE0204400

## Abstract

Biosensors can be a powerful tool for real-time monitoring of specific small molecules and for precise control of gene expression in biological systems. Thus, biosensors have attracted much attention for monitoring increasing number of molecules. However, strategies to tune the properties of biosensors remain less explored, which might restrict their wide applicability. Here we report the development of tunable biosensors for monitoring putrescine, an important member of biological polyamines, in *Escherichia coli*. The native putrescine-responsive PuuR repressor protein was employed as a sensing component, and its cognate operator was installed in engineered promoters to control the expression of downstream green fluorescent protein (GFP) mut3 as a reporter protein. The engineered biosensors were specific for putrescine, and the response time could be modulated by altering growth medium of the biosensor strains. In addition, the response dynamics and detection ranges of the biosensors can be tuned at the genetic level by modulation of PuuR expression, and by manipulation of the chromosomal genes involved in putrescine biosynthesis. To demonstrate utility of the biosensors, we were able to monitor the changes of endogenous putrescine levels caused by genetic manipulations. Furthermore, a link between the excretory putrescine titer and intracellular GFP fluorescence was established for an *E. coli* strain that was engineered for improved putrescine biosynthesis and excretion. This study provides a strategy for engineering synthetic biosensor circuit for monitoring and tuning the dynamics in sensing putrescine, which can be generally applicable for monitoring other chemicals through taking a similar approach in circuit design.

**KEYWORDS**
biosensor, *Escherichia coli*, putrescine, PuuR, response dynamics, synthetic biology

## 1 | INTRODUCTION

In nature, living biological systems are evolved with sophisticated capabilities to sense a broad spectrum of small molecules and regulate genetic expression at hierarchical levels (Roth & Breaker, 2009; Stanton et al., 2014). The specific sensing is achieved, in part, through small molecule-responsive transcription factors and their cognate promoters (Stanton et al., 2014). In another scenario, RNA riboswitches known as noncoding *cis*-regulatory elements found in the 5′-untranslated regions of mRNAs, bind intracellular small-molecule metabolites through aptamer domains and convert the metabolic signal into a change in translation of downstream mRNA sequences

  

(Roth & Breaker, 2009). Inspired by nature, scientists have recently begun to explore, recruit, and even repurpose the sensing components for the construction of genetically encoded biosensors for in situ monitoring of small molecules (Mahr & Frunzke, 2016; Skjoedt et al., 2016; Zhang, Jensen, & Keasling, 2015; Zhou & Zeng, 2015).

Indeed, biosensors are emerging as powerful devices with diverse applications in synthetic biology and metabolic engineering (Liu, Evans, & Zhang, 2015; Zhang et al., 2015). For instance, the tetracycline-responsive TetR biosensors have for decades been used for precise control of gene expression in both prokaryotic and eukaryotic systems (Ramos et al., 2005; Smanski et al., 2016). Recently, metabolite biosensors have been developed and used to monitor product formation at the single cell level in real-time (Rogers & Church, 2016) and to identify high-performance enzymes in biosynthetic pathways and superior transporters for sugar uptake (Schendzielorz et al., 2014; Tang et al., 2013; Wang, Li, & Zhao, 2016). In addition, metabolite biosensors have been demonstrated to enable high-throughput screening of microbial overproducers by programming adaptive control over production phenotypes (Chou & Keasling, 2013), by imbuing a fitness advantage to overproducing cells (Raman, Rogers, Taylor, & Church, 2014; Yang et al., 2013), by exploiting nongenetic cell-to-cell variation (Xiao, Bowen, Liu, & Zhang, 2016), and by dynamic, tunable overexpression of multiple enzymes of the biosynthetic pathways (Dahl et al., 2013; Dietrich, Shis, Alikhani, & Keasling, 2013; Fang et al., 2016; Xu, Li, Zhang, Stephanopoulos, & Koffas, 2014; Zhang, Carothers, & Keasling, 2012). These developments highlight the exciting opportunities for the development of genetically encoded biosensors for broader applications.

Transcription factor-based biosensors are a family of sensing devices that have received most wide attention (Skjoedt et al., 2016; Stanton et al., 2014). This seems to be due to the fact that microbial genomes are a rich source of transcription factors that control biosynthesis of numerous products including amino acids, nucleotides, and vitamins (Skjoedt et al., 2016; Stanton et al., 2014). One approach to construct transcription factor-based biosensors is to directly recruit the natural transcriptional repressors or activators for the recognition of specific metabolites (Moser, Horwitz, Chen, Lim, & Voigt, 2013). Alternatively, the natural transcriptional factors can be engineered at the protein level to recognize non-native metabolites. For example, the L-arabinose-responsive transcriptional regulator AraC from *Escherichia coli* has been engineered to specifically respond to the levels of D-arabinose (Tang, Fazelinia, & Cirino, 2008), triacetic acid lactone (Tang et al., 2013), and mevalonate (Tang & Cirino, 2011). Another strategy to expand the range of biologically detectable molecules is to transform nondetectable molecules via multistep biochemical reactions into molecules for which sensors already exist (Libis, Delépine, & Faulon, 2016). Therefore, the range of molecules that can be monitored has been expanding rapidly and now includes certain members of sugars, amino acids (Mahr & Frunzke, 2016; Mustafi, Grünberger, Kohlheyer, Bott, & Frunzke, 2012), lactams (Zhang et al., 2017), organic acids (Li & Yu, 2015), redox molecules (Zhang et al., 2016), heavy metals (Bereza-Malcolm, Mann, & Franks, 2015; Cerminati, Soncini, & Checa, 2011), organophosphates (Chong & Ching, 2016), and flavonoids (Siedler, Stahlhut, Malla, Maury, & Neves, 2014). However, until now, there is no report on the development of biosensors for monitoring polyamines, which are ubiquitous organic cations of low molecular weight found in a wide range of organisms with important physiological roles (Tabor & Tabor, 1985).

Response dynamics is an important feature of biological sensing systems (Ang, Harris, Hussey, Kil, & McMillen, 2013). Although a variety of transcription factor-promoter pairs are readily available by genomic mining (Stanton et al., 2014), these sensor devices are naturally adapted to the organisms' physiological purposes and usually limited in response characteristics such as specificity, sensitivity, detection range, and response time (Mahr & Frunzke, 2016). Therefore, the natural transcription factors and their cognate promoters need to be engineered either individually or in combination for tuning the responses of the resulting biosensor systems. Due to the highly modular architecture, the promoters can be rewired by altering the sequence, position, and number of operators, with which the transcription factors bind. For example, the dynamic range of an oleic acid biosensor was twelvefold higher by replacing the native FadR-regulated *fadBA* promoter with a synthetic promoter, which was designed by the introduction of two copies of FadR-binding operator sequence into the strong phage *T7* promoter (Zhang et al., 2012). More recently, impact of mutations to Lac operator site on isopropyl β-D-1-thiogalactopyranoside biosensors was examined, which revealed interdependencies between biosensor dynamic range and response threshold (Mannan, Liu, Zhang, & Oyarzún, 2017). Another strategy to modulate the response of a biosensor is altering the expression levels of transcription factors (Liu, Evans, & Zhang, 2015; Wang et al., 2016). This was elegantly demonstrated by adjusting the amount of FapR under the control of an inducible $P_{BAD}$ promoter to modulate the response curves of a malonyl-CoA biosensor (Liu, Xiao, Evans, & Zhang, 2015). Although much work has been done, the strategies to tune the properties of biosensors remain less explored, particularly for monitoring metabolites that are involved in complex metabolic networks, including biosynthesis, degradation, uptake, and export.

Putrescine, also known as 1,4-diaminobutane, is a crucial intermediate in polyamine metabolism with important roles in cell proliferation and normal cell growth (Tabor and Tabor, 1985). Also, it is an important four carbon diamine monomer for the production of engineering plastics (Jang et al., 2012; Nguyen, Schneider, Reddy, & Wendisch, 2015; Qian, Xia, & Lee, 2009). As a first step to monitor diverse polyamines in living organisms, here we report the development of biosensors based on the transcriptional factor PuuR for monitoring putrescine level in *E. coli*. First, synthetic promoters were designed and evaluated based on their ability to respond to PuuR and putrescine. Various strategies were then explored to modulate the responsive properties of the putrescine biosensors. Finally, the biosensors were utilized for monitoring intracellular putrescine levels upon modifications on putrescine biosynthetic capacity, and for monitoring putrescine production.

## 2 | MATERIALS AND METHODS

### 2.1 | Chemicals and materials

1,3-Diaminopropane, putrescine, 1,5-diaminopentane dihydrochloride, and 1,6-diaminohexane were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). All other chemical reagents were of the highest purity commercially available. PrimeSTAR Max DNA polymerase was obtained from Takara Biotechnology Co., Ltd (Dalian, China). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA). Mini Plasmid purification Kit, Bacteria DNA Kit, PCR Purification Kit, and gel Purification Kit were purchased from TIANGEN Biotech (Beijing, China).

### 2.2 | Plasmids and strains

Plasmids constructed in this study are listed in Table 1. PCR primers used for plasmid construction are listed in Table 2. A description of plasmids construction, deletion of chromosomal genes, promoter replacement, and gene integration into the *E. coli* chromosome is provided in Supplementary Methods.

### 2.3 | Characterization of puuR-responsive promoters

To characterize the native *puuA* and synthetic promoters, each of the *gfpmut3*-expressing plasmids was transformed into the *puuR* knockout strain and the *speC speB speF* triple mutant. The former strain allowed full "ON" transcription of GFPmut3 due to PuuR absence, while the latter permitted only basal expression due to the presence of PuuR and absence of putrescine. These recombinant strains were inoculated into a 15 ml tube containing 4 ml of LB medium with 50 μg $ml^{-1}$ of kanamycin (Km), and grown overnight at 37°C and 220 rpm in a rotary shaker. The overnight cultures were then diluted 100-fold into 200 μl of fresh LB medium on a 96-well cell culture plate (Flat bottom; Nest Biotech Co., Ltd, Wuxi, China). The plate was incubated and humidified at 37°C and 400 rpm for 20 hr. Cell growth ($OD_{600}$) and fluorescence (excitation 488 nm, emission 520 nm) were measured with a Spectramax M5 plate reader (Molecular Devices, Sunnyvale, CA). All fluorescence measurements were background corrected and normalized by $OD_{600}$. The dynamic range for each promoter was determined by dividing the fluorescence obtained in the *puuR* knockout strain by the level of fluorescence observed in the triple mutant.

### 2.4 | Characterization of biosensor strains

Biosensor specificity was studied for *E. coli* MG1655 harboring pTacR2gfp with LB medium at 37°C on the 96-well cell culture plates as described above. Each of the C3 to C6 diamines tested was first dissolved in fresh LB medium, adjusted to pH 7.0 with concentrated hydrochloric acid or 6 M NaOH, and 0.22-μm membrane sterilized to prepare stock solutions. Briefly, the recombinant strain was first inoculated into a test tube and grown overnight. The overnight culture (0.5 ml) was subsequently transferred into a 250-ml Erlenmeyer flask containing 50 ml of LB medium at 37°C and 220 rpm. When the cell $OD_{600}$ reached 0.3–0.4, this preculture (100 μl) was mixed in each well of the plates with an equal volume of pre-warmed LB medium supplemented with one of the diamines from the stock solutions. The cells exposed to each of the diamines at a final concentration of 0.01–50 mM were incubated at 37°C and 400 rpm for 20 hr. $OD_{600}$ and GFP fluorescence were then measured using the Spectramax M5 plate reader as described above.

Response kinetics was studied for *E. coli* MG1655 harboring pTacR2gfp upon exposure to putrescine in three different culture media: LB medium, M9 medium, and a modified M9 medium. The M9 medium contained (in g $L^{-1}$): $Na_2HPO_4 \cdot 7H_2O$, 12.8; $KH_2PO_4$, 3; NaCl, 0.5; $NH_4Cl$, 1; $MgSO_4$, 0.241; $CaCl_2$, 0.011; and glucose, 4. The modified M9 medium contained the same components as the M9 medium except that $NH_4Cl$ was omitted. Putrescine was first dissolved in each of the above three media, adjusted to pH 7.0, and filter sterilized to make working solutions before being mixed with the bacterial cells. For response kinetic study with LB medium, the cell preculture was prepared in the same manner as in the specificity test described above. For studies with the M9 and modified M9 medium, the recombinant strain was first grown overnight in LB medium, transferred into a 250-ml Erlenmeyer flask containing 50 ml of M9 medium at 37°C and 220 rpm, and grown to an $OD_{600}$ of 0.3–0.4 as the preculture for test with M9 medium. In another experimental setup, the cells were harvested by centrifugation at 3,214*g* and 25°C for 10 min, washed twice with the modified M9 medium, re-suspended with the same volume of fresh medium, and then used as the preculture. The cells exposed to putrescine at a final concentration of 6 mM were grown on the plates for 24 hr, with $OD_{600}$ and GFP fluorescence monitored every 2 hr using the plate reader.

Dose-response studies were performed for the biosensor strains with genetically modified backgrounds in both LB and the modified M9 medium. Cell precultures and putrescine stock solutions were similarly prepared as described above. The cells exposed to putrescine at final concentrations from 0.001 mM up to 100 mM were incubated on the plates at 37°C. Following putrescine exposure, $OD_{600}$ and GFP fluorescence were determined for the cells grown with the modified M9 medium and LB medium at 10 and 20 hr, respectively.

### 2.5 | Putrescine biosynthesis and overproduction in flask cultivation

*E. coli* MG1655 and its derivatives with genetic manipulations on the putrescine biosynthetic genes were transformed with pTacR2gfp. Each recombinant strain was grown with LB medium in a tube overnight at 37°C, diluted 1:100 into a flask containing 50 ml of M9 medium, and incubated at 37°C and 220 rpm to an $OD_{600}$ of 0.8–1.0, with GFP fluorescence monitored by the plate reader. An aliquot of fermentation broth corresponding to ~$3*10^9$ cells was centrifuged at 15,557*g* and 4°C for 10 min, and the resulting cell pellets were used for quantification of intracellular putrescine levels.

To establish a link between intracellular GFP intensities and extracellular putrescine titers, an *E. coli* MG1655-derived putrescine overproducer, strain ZQ19 was constructed (see Supplementary Method for details). *E. coli* ZQ19 harboring pTacR2gfp was first grown in LB

**TABLE 1** *Escherichia coli* strains and plasmids used in this study

| Strain/plasmid | Description[a] | Source or reference |
|---|---|---|
| Strains | | |
| TOP10 | F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ¢80*lac*ZΔ M15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)*7697* *gal*U *gal*K *rps*L (Str$^R$) *end*A1 *nup*G | Invitrogen[b] |
| DH5α | F⁻ φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 ($r_k^-$ $m_k^+$) *pho*A *sup*E44 λ⁻ *thi*-1 *gyr*A96 *rel*A1 | Invitrogen[b] |
| MG1655 | *Coli* Genetic Stock Center strain (CGSC) No. 6300 | CGSC[c] |
| XF07C | MG1655 *puuR*::Cm$^R$ | This study |
| XF18C | MG1655 *speB*::Cm$^R$ | This study |
| XF19C | MG1655 *speF*::Cm$^R$ | This study |
| XF20C | MG1655 *speC*::Cm$^R$ | This study |
| XF52 | MG1655 Δ*speC* Δ*speB* | This study |
| XF56 | MG1655 Δ*speC* Δ*speB* Δ*speF* | This study |
| ZQ09 | MG1655 Δ*puuAP* Δ*speG* Δ*speE* Δ*ygjG* | This study |
| ZQ19 | ZQ09 P*speF-potE*::$P_{N25}$ | This study |
| ZQ26 | ZQ09 *lacI*::$P_{N25}$-*speC*' | This study |
| Plasmids | | |
| pKD46 | Ap$^R$, λ Red recombinase under arabinose-inducible *araBAD* promoter, *ts* origin, 6.3-kb | Datsenko and Wanner (2000) |
| pKD3 | Ap$^R$, Cm$^R$, FRT-Cm$^R$-FRT cassette, 2.8-kb | Datsenko and Wanner (2000) |
| pZS*26mCherryFRT | Km$^R$, Cm$^R$, FRT-Cm$^R$-FRT, *mCherry* under $P_{N25}$ promoter, pSC101* origin, 5.2-kb | Lab stock |
| pZS*26speCFRT | Km$^R$, Cm$^R$, FRT-Cm$^R$-FRT, *E. coli speC* under $P_{N25}$ promoter, pSC101* origin, 6.7-kb | This study |
| pZS*26speC'FRT | Km$^R$, Cm$^R$, FRT-Cm$^R$-FRT cassette, variant *speC* under $P_{N25}$ promoter, pSC101* origin, 6.7-kb | This study |
| pECA102 | Ap$^R$, Flp recombinase under arabinose-inducible *araBAD* promoter, *sacB* cassette, 3.5-kb | Dr. D. E. Cameron |
| pZA2pBAD-gfp | Km$^R$, p15A ori, *gfpmut3* under arabinose-inducible *araBAD* promoter, 2.8-kb | Dr. D. E. Cameron |
| pZA27gfp | Km$^R$, p15A ori, *gfpmut3* under constitutive *lacIQ* promoter, 2.9-kb | This study |
| ppuuAgfp | Km$^R$, p15A ori, *gfpmut3* under *puuA* promoter, 2.9-kb | This study |
| pAR2gfp | Km$^R$, p15A ori, *gfpmut3* under *AR2* promoter, 2.9-kb | This study |
| pAR3gfp | Km$^R$, p15A ori, *gfpmut3* under *AR3* promoter, 2.9-kb | This study |
| pLR2gfp | Km$^R$, p15A ori, *gfpmut3* under *LR2* promoter, 2.9-kb | This study |
| pLR3gfp | Km$^R$, p15A ori, *gfpmut3* under *LR3* promoter, 2.9-kb | This study |
| pTacR2gfp | Km$^R$, p15A ori, *gfpmut3* under *TacR2* promoter, 2.9-kb | This study |
| pTacR3gfp | Km$^R$, p15A ori, *gfpmut3* under *TacR3* promoter, 2.9-kb | This study |
| pZA27puuR | Km$^R$, p15A ori, *E. coli puuR* under *lacIQ* promoter, 2.7-kb | This study |
| pS6 | Km$^R$, p15A ori, *gfpmut3* under *TacR2* promoter, *puuR* under *lacIQ* promoter, 3.6-kb | This study |

[a]Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Str, streptomycin; R, resistance; ts, temperature sensitive.
[b]Invitrogen, Corp., Carlsbad, CA.
[c]Coli Genetic Stock Center, New Haven, CT.

overnight, and then diluted 1:100 into a flask containing 50 ml of R/2 medium supplemented with 10 g $L^{-1}$ of glucose and 3 g $L^{-1}$ of $(NH_4)_2SO_4$ (Qian et al., 2009; Qian, Xia, & Lee, 2011). The cells were subsequently incubated at 220 rpm for 12 hr, when glucose was completely consumed. Culture broth (200 µl) was taken every 2 hr for measurements of GFP fluorescence on the 96-well plates. Upon centrifugation at 13,523*g* and 4°C for 10 min, the culture supernatants were collected and stored at −20°C for analyses of extracellular putrescine concentrations by HPLC. For accurate measurement, the samples were diluted, when necessary, to give putrescine concentration of less than 50 mg $L^{-1}$. All the experiments were carried out in triplicates.

## 2.6 | Quantification of putrescine levels by HPLC

The cell pellets for intracellular putrescine quantification were washed once in 50 mM phosphate buffer (pH 7.0) and centrifuged at 13,523*g* and 4°C for 10 min. The pellets were resuspended in the above phosphate buffer and lysed with an Ultrasonic Homogenizer (Model

**TABLE 2** PCR primers used in this study.

| Primer | Sequence (5′ to 3′)[a] |
|---|---|
| FPiq | TCGAGTGGTGCAAAACCTTTCGCGGTATGGCATGATAGCGCCCGGAAGAGAGTCAATTCAGGG |
| RPiq | AATTCCCTGAATTGACTCTCTTCCGGGCGCTATCATGCCATACCGCGAAAGGTTTTGCACCAC |
| FpuuApXh1 | CCCGCTCGAGTCATTTTTGCAAACTCAATTTA |
| RpuuApKp | CACTGGTACCGATTCTTCGCCTTTGGTTT |
| FpAR2Xh | TCGAGAAAATTTATCAAAAAGAGTGTTGACTGTGGTCATTATATTTTACGCGATACTTAGATTCAGTGGTCATTATATTTTACGCG |
| RpAR2Ec | AATTCGCGTAAAATATAATGACCACTGAATCTAAGTATCGCGTAAAATATAATGACCACAGTCAACACTCTTTTTGATAAATTTTC |
| FpAR3Xh | TCGAGAAAATTTATCAAAAAGAGTGTTGACTTAAAGTCTAACCTATAGGATACTTAGATTCAGTGGTCATTATATTTTACGCG |
| RpAR3Ec | AATTCGCGTAAAATATAATGACCACTGAATCTAAGTATCCTATAGGTTAGACTTTAAGTCAACACTCTTTTTGATAAATTTTC |
| FpLR2Xh | TCGAGATAAATTATCTCTGGCGGTGTTGACAGTGGTCATTATATTTTACGCGATACTGAGCACAGTGGTCATTATATTTTACGCG |
| RpLR2Ec | AATTCGCGTAAAATATAATGACCACTGTGCTCAGTATCGCGTAAAATATAATGACCACTGTCAACACCGCCAGAGATAATTTATC |
| FpLR3Xh | TCGAGATAAATTATCTCTGGCGGTGTTGACATAAATACCACTGGCGGTGATACTGAGCACAGTGGTCATTATATTTTACGCG |
| RpLR3Ec | AATTCGCGTAAAATATAATGACCACTGTGCTCAGTATCACCGCCAGTGGTATTTATGTCAACACCGCCAGAGATAATTTATC |
| FptacR2Xh | TCGAGCTGTTGACAGTGGTCATTATATTTTACGCTATAATGTGTGGAGTGGTCATTATATTTTACGCG |
| RptacR2Ec | AATTCGCGTAAAATATAATGACCACTCCACACATTATAGCGTAAAATATAATGACCACTGTCAACAGC |
| FptacR3Xh | TCGAGCTGTTGACAATTAATCATCGGCTCGTATAATGTGTGGAGTGGTCATTATATTTTACGCG |
| RptacR3Ec | AATTCGCGTAAAATATAATGACCACTCCACACATTATACGAGCCGATGATTAATTGTCAACAGC |
| FpuuRKp | CGGGGTACCATGAGTGATGAGGGACTGGC |
| RpuuRXm | TCCCCCCGGGTTAAAACGTGGTGGGCGTAT |
| F7puuRTAa | TTAGGACGTCTCTAGGGCGGCGGATTTGTC |
| R7puuRTXh | GACAGCTCGAGTGGTGCAAAACCTTTCG |
| FKOpuuR | GCGACTCTGACCACTACAGTTTAAGGAAATGCAAATATGTGTGTAGGCTGGAGCTGCTT |
| RKOpuuR | GCGCATTGCGCTTACCCGGCTTACAGAACAAAAAGATTAGGGAATTAGCCATGGTCCAT |
| FKOspeC | GGTATTCTTACTTCCCCGAAACGGGTTTGCGCTTATGTGTAGGCTGGAGCTGCTT |
| RKOspeC | AAACGGGTCGCCAGAAGGTGACCCGTTTTTTTTATTCTTAGGGAATTAGCCATGGTCCAT |
| FKOspeF | TGAGGACCTGCTATTACCTAAAATAAAGAGATGAAAAATGTGTGTAGGCTGGAGCTGCTT |
| RKOspeF | TTAACTGAACGACGCCCATTTTGTTCGATTTAGCCTGACTCAGGGAATTAGCCATGGTCCAT |
| FKOspeB | CGCGGAAGGGTTTTTTTATATCGACTTTGTAATAGGAGTCCATCCTGTGTAGGCTGGAGCTGCTT |
| RKOspeB | AATGGCACGTTTTACCCGTGCGCATCGCATCTGGTGCTTAGGGAATTAGCCATGGTCCAT |
| FKOspeB2 | CCGCCACAATTTATTGTGACAAATCCAACCCTTCCTCGTCGGGCCTAACGACGCGGAAGGGTTTTTTTATA |
| RKOspeB2 | ACGGGTGCCGAACGTAGGTCGGATAAGGCGTTCACGCCGCATCCGACATTAATGGCACGTTTTACCCGTG |
| FKOspeF2 | CGGGGTAACTTTGCTTTTTTCCGGCACGATCGATTTCTCATTCGAGAAATTGAGGACCTGCTATTACCTA |
| RKOspeF2 | CGGCAGCATGATGATACCGGAGCCCATCATGTTGACCATCGTCAGTATGGTTAACTGAACGACGCCCATT |
| FKOpuuA | TGATAACGAGCGGAAAACAAACCAAAGGCGAAGAATCATGTGTGTAGGCTGGAGCTGCTT |
| RKOpuuP | TGCGCCGCGCATCCGACTATTACGTTTCACTCACCGGCGTGGGAATTAGCCATGGTCCAT |
| FKOspeG | AACCTGTTATTGATTTAAGGAATGTAAGGACACGTTATGTGTGTAGGCTGGAGCTGCTT |
| RKOspeG | TTACACCATCAAAAATACGATCGATTATTATTAATGCTAGGGAATTAGCCATGGTCCAT |
| FKOspeE | TTTTTTTACGGGTGTTAACAAAGGAGGTATCAACCCATGTGTGTAGGCTGGAGCTGCTT |
| RKOspeE | ATGCAGTTTCAGTTTTTTCAATTTCTTATCTTCTCCTTAGGGAATTAGCCATGGTCCAT |
| FKOygjG | CGGTATCATGTGATACGCGAGCCTCCGGAGCATATTTTGTGTGTAGGCTGGAGCTGCTT |
| RKOygjG | TCGGATGGCGACGTCGTATCGCCATCCGATTTGATATTAGGGAATTAGCCATGGTCCAT |
| FPspeF | TAAGGGCACTTCAGCGTACAGGTCTTCCTGACTCTCTGTAGGCTGGAGCTGCTT |
| RPspeF | GTCCGGGCAAGAATCACTAACCGCAATTTTTAATTTTGACATGGTACCTTTCTCCTCTTT |
| FlacIFRT | GTGAAACCAGTAACGTTATACGATGTCGCAGAGTATGCCGGTGTCTCTTATGTGTAGGCTGGAGCTGCTT |
| RlacIT1 | TCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGGCGGATTTGTCCTACTCAGG |

(Continues)

**TABLE 2** (Continued)

| Primer | Sequence (5′ to 3′)[a] |
|---|---|
| FspeCKpn | CCTCGGTACCATGAAATCAATGAATATTGCC |
| FspeCBam | AATCGGATCCTTACTTCAACACATAACCGTA |
| FspeC' | ACCCATACCGGATCGGCGAAAGAT |
| RspeC' | AAGCAGATCGCCCAATTTTACGTCAGC |

[a]Restriction sites are underlined.

JY92-IIDN; Scientz, Ningbo, China). Upon centrifugation, the cell-free lysates were filtered through a 0.22-μm membrane for quantification of putrescine levels. Putrescine concentration was determined by precolumn *o*-phthaldialdehyde derivatization coupled with reverse-phase HPLC and UV detection at 230 nm, which was essentially same as described in our previous study (Qian et al., 2009). Intracellular putrescine level was normalized by biomass dry weight (DW) concentration, which was estimated based on the assumption that one $OD_{600}$ unit equals 0.33 g DW $L^{-1}$ of *E. coli* cell cultures. The data represent the average of three biological replicates and error bars correspond to the standard deviations.

## 2.7 | Data fitting

Putrescine response curves were fitted to the Hill equation as follows:

$$y = \mathrm{GFP}_{\min} + \frac{(\mathrm{GFP}_{\max} - \mathrm{GFP}_{\min})\cdot x^n}{k^n + x^n}$$

Here, *y* denotes the normalized fluorescence value corresponding to the putrescine concentration level at *x*. *k* represents the putrescine concentration resulting in half-maximal induction, and *n* is the Hill coefficient. $\mathrm{GFP}_{\min}$ and $\mathrm{GFP}_{\max}$ represent the smallest baseline and largest fluorescent signals, respectively. These parameters can be estimated by fitting the experimental data by using OriginPro 8 software (OriginLab Corporation, Northhampton, MA) according to the manufacturer's instructions. Non-linear least-squares regression was used to minimize error between the fitted and actual data. The lower limit of detection was then estimated, which corresponded to the putrescine concentration resulting in a GFP signal higher than mean $\mathrm{GFP}_{\min}$ by three times of the standard deviation. The upper limit of detection was also estimated, which represented the putrescine concentration resulting in a GFP signal lower than mean $\mathrm{GFP}_{\max}$ by three times of the corresponding standard deviation.

To link extracellular putrescine titer and intracellular GFP intensity for the putrescine overproducer in shake flask fermentation, a mathematical model was developed. As the GFP intensity was approaching a final stable level in a nonlinear manner, an asymptotic function was proposed for data fitting as follows:

$$y = \mathrm{a} - \mathrm{b}\cdot c^x$$

Here, *y* represents the intracellular GFP intensity at the time point when extracellular putrescine titer was at *x*. The parameters of the asymptotic function have clear biological relevance. The *asy* (a) is the maximal fluorescence value on continuous secreting putrescine, the *int* (a-b) is the fluorescence value at initiation of excretory putrescine production (beginning of flask fermentation). The constant *c* characterizes the rate of increase in fluorescence intensity, with $-\log_c(2)$ being the excretory titer for half the ultimate increase in fluorescent from *int* to *asy* to occur.

# 3 | RESULTS

## 3.1 | Biosensor design

As a potential transcription factor for sensing putrescine, PuuR is a negative regulator of the Puu pathway comprising the enzymes encoded by the *puu* genes that metabolize putrescine via γ-glutamylated intermediates to γ-amino butyric acid in *E. coli* K12 (Kurihara et al., 2005; Nemoto et al., 2012). It has been revealed that PuuR has a helix-turn-helix motif that binds to the promoter region of the first gene (*puuA*) of the Puu pathway (Nemoto et al., 2012). As putrescine responsive PuuR and its cognate promoter are naturally existing in *E. coli*, we decided to design and construct tunable PuuR-based biosensors in *E. coli* (Figure 1). The cognate promoter positioned upstream of the green fluorescent protein reporter gene (*gfpmut3*) could serve as a PuuR-repressive genetic element to express green fluorescence in a manner that depended on the intracellular concentration of putrescine. By manipulation of the expression level of PuuR, its cognate promoter and the chromosomal genes involved in putrescine biosynthesis, the resulting putrescine biosensors are anticipated to exhibit tunable properties for monitoring external putrescine.

## 3.2 | Design and evaluation of synthetic responsive promoters

For use as an initial biosensor, plasmid ppuuAgfp expressing the *gfpmut3* gene under control of the native *puuA* promoter of *E. coli* (Figure 2a) was constructed. The promoter activity was evaluated under two genetic backgrounds, both of which were derived from wild-type *E. coli* K12 MG1655. One was defective in the chromosomal *puuR* gene to mimic fully derepressed state for the *puuA* promoter, whereas the other harbored intact *puuR* but deficient in all the known genes responsible for putrescine biosynthesis, which would reveal basal activity of the *puuA* promoter under repressed state (Figure 2b); the *puuR* single knockout strain XF07C and the *speC speB speF* triple

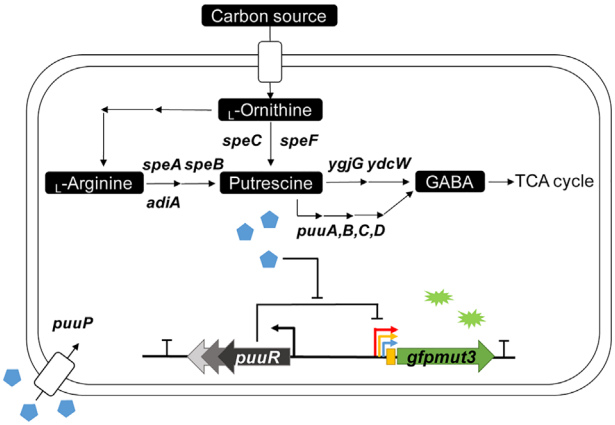


**FIGURE 1** Schematic representation of the development of tunable putrescine biosensors in *E. coli*. *puuR* is expressed at diverse levels, from either chromosome or plasmid-based overexpression. PuuR represses *gfpmut3* transcription from a synthetic PuuR-responsive promoter of suitable strength, and the repression is relieved upon binding of PuuR with putrescine, thus generating GFP signal in a putrescine dose-dependent manner. The metabolic pathways involved in putrescine biosynthesis, uptake and catabolism were also shown. Enzymes encoded by the genes are: *adiA*/*speA*, degradative/biosynthetic arginine decarboxylase; *puuA*, glutamate-putrescine ligase; *puuB*, γ-glutamylputrescine oxidase; *puuC*, γ-glutamyl-γ-aminobutyraldehyde dehydrogenase; *puuD*, γ-glutamyl-γ-aminobutyrate hydrolase; *puuP*, putrescine importer; *puuR*, transcription factor of Puu pathway; *speB*, agmatinase; *speC*/*speF*, biosynthetic/degradative ornithine decarboxylase; *ydcW*, γ-aminobutyraldehyde dehydrogenase; *ygjG*, putrescine aminotransferase. Abbreviations: GABA, γ-aminobutyrate; TCA cycle, tricarboxylic acid cycle [Color figure can be viewed in the online paper, which is available at http://onlinelibrary.wiley.com/doi/10.1002/bit.26521/epdf]

knockout strain XF56 were thus constructed. Strains XF07C and XF56 harboring plasmid ppuuAgfp were then grown in standard LB medium at 37°C and cell culture fluorescence (normalized by cell density) was measured. GFP fluorescence outputs of the *puuA* promoter from both XF07C and XF56 were ~50% higher than the respective autofluorescence levels without GFP expression (Figure 2c), indicating only marginal activity of the *puuA* promoter in both the putrescine deficient and the PuuR defective backgrounds. On the other hand, the dynamic range of this promoter, represented as the ratio of fluorescence at the derepressed state to the repressed state, was rather low (1.24). For proper evaluation of the dynamic range, a moderate, constitutive *lacIQ* promoter was also included in the assay. Even though the *lacIQ* promoter was not supposed to respond to PuuR, this promoter showed a 1.22-fold change in fluorescence output. Therefore, the native *puuA* promoter was undesirable and the creation of PuuR-regulated synthetic promoters with better sensitivity was necessary.

Next, we designed a series of synthetic promoters by inserting PuuR operator sequence into strong promoters, such as the *tac*, phage lambda$_{\cdot}$ and phage *T7* promoters (Lutz & Bujard, 1997). Earlier, the PuuR operator (PuuO) with a length of 20 bp has been identified from the *puuA* promoter region of *E. coli* (Nemoto et al., 2012). In order to examine the effects of varying the copy number and position of PuuO, synthetic promoters were made with either a single PuuO downstream of transcriptional start site or with an additional one between the −35 and the −10 region of the phage *T7*, phage lambda and *tac* promoters; the *AR2*, *LR2*, and *TacR2* promoters, each with dual PuuO sequences, and the single PuuO-harboring *AR3*, *LR3*, and *TacR3* promoters were thus constructed (Figure 2a). The engineered promoters were then placed upstream of the *gfpmut3* reporter gene and evaluated in *E. coli* XF07C and XF56. GFP fluorescence levels from the synthetic promoters with a single PuuO were approximately 30–100-fold higher than the promoters with double PuuO in both strains XF07C and XF56 (Figure 2c). This result indicates that the additional PuuO placed between the −35 and the −10 region of synthetic promoters can serve as an efficient transcription roadblock. Notably, the *TacR2* promoter was found to exhibit the highest dynamic range of 3.69 (see section 2 for dynamic range), whereas the remaining five synthetic promoters showed a dynamic range of 1.21–1.71. Based on these results, plasmid pTacR2gfp harboring the *TacR2* promoter upstream of the *gfpmut3* reporter was selected for its use in biosensor construction described below.

## 3.3 | Putrescine biosensors are highly specific

We next studied specificities of the PuuR-based putrescine biosensors. This was performed with a typical sensing strain *E. coli* MG1655 harboring the chromosome-encoded PuuR and the biosensor plasmid pTacR2gfp. Three biologically relevant diamines, 1,3-diaminopropane, putrescine (1,4-diaminobutane) and cadaverine (1,5-diaminopentane) and a non-natural diamine 1,6-diaminohexane were included in the specificity test. To this end, exponentially growing cells of *E. coli* MG1655 (pTacR2gfp) were treated with each of the above linear chain C3 to C6 diamines at a final concentration from 0 to 50 mM. As shown in Figure 3, GFP fluorescence of the biosensor strain responded to putrescine in a dose-dependent manner. The fluorescence displayed at background level without putrescine treatment (data not shown), started to increase with 0.1 mM putrescine, and reached a 6.8-fold higher level upon exposure to 50 mM putrescine. However, none of the other three diamines triggered any significant increase in the fluorescence signal over the entire concentration range tested (0.01–50 mM). These results demonstrated that the whole-cell biosensor was specific for putrescine over 1,3-diaminopropane, cadaverine and 1,6-diaminohexane.

## 3.4 | Modulation of response time by altering culture medium

We then explored whether the response time of putrescine whole-cell biosensor could be modulated. It was hypothesized that cell culture conditions, particularly nutrients in medium, might affect uptake of exogenous input molecule and thus response time by the whole-cell biosensor. To test this hypothesis, the putrescine biosensor, *E. coli* MG1655 (pTacR2gfp) was exposed to 6 mM putrescine in the rich LB medium, glucose minimal M9 medium, and a modified M9 medium without $NH_4Cl$ (Figure 4). During the first 6 hr upon putrescine

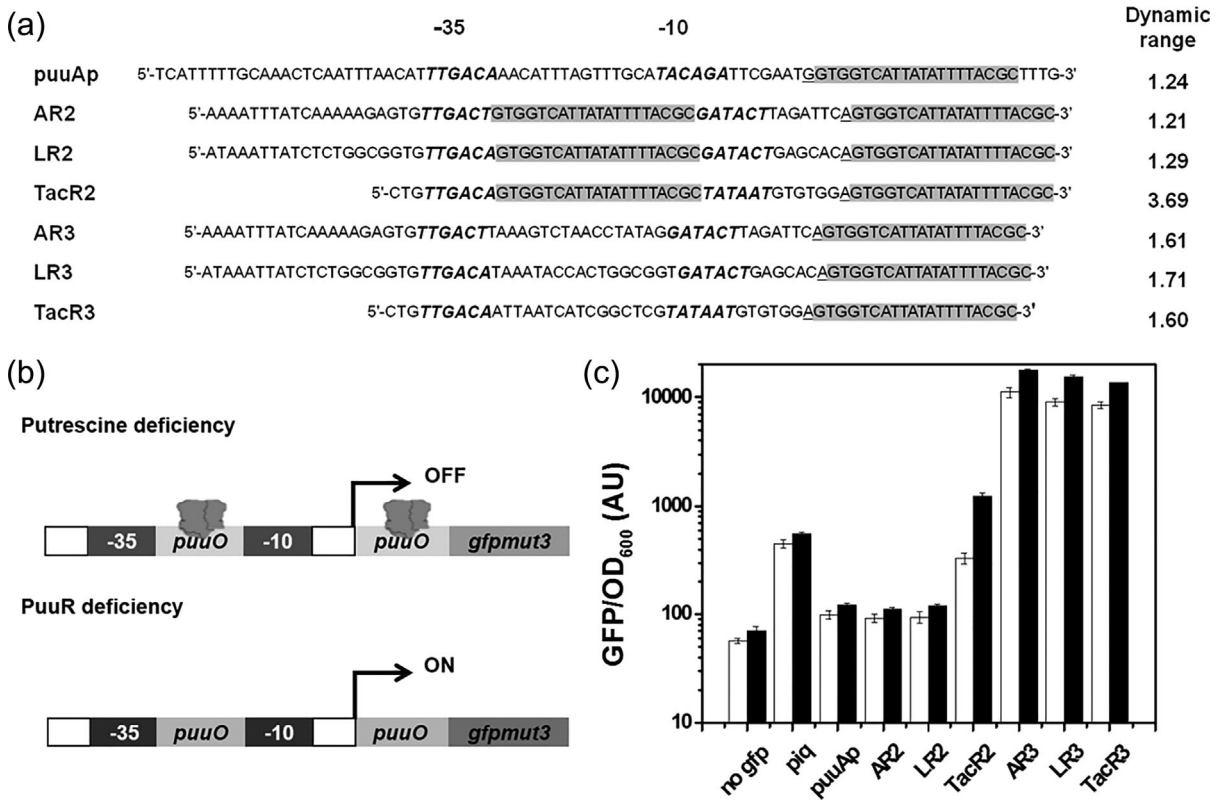


**FIGURE 2** Design and evaluation of synthetic PuuR-responsive promoters. (**a**) DNA sequence of synthetic promoters compared with the native *puuA* promoter (*puuAp*). The sequences in italics represent the −10 and −35 regions. The PuuR recognition site is in grey and transcriptional start site underlined. The dynamic range of each promoter was also shown. (**b**) In the absence of intracellular and environmental putrescine, PuuR binds to the operator sequence (*puuO*) and represses *gfpmut3* transcription. When PuuR is absent, *gfp* transcription is fully "ON," regardless the presence of putrescine. (**c**) The strength of each promoter at the repressed and fully "ON" states. Plasmid harboring each *gfpmut3*-reporting promoter was transformed into *E. coli* MG1655 with deficiency either in putrescine biosynthesis (open column) or in PuuR (filled column). Cells were grown in LB medium at 37°C and fluorescence measured at 20 hr. The dynamic range of each promoter was determined by dividing the fluorescence observed in the *puuR* knockout strain (XF07C) by that in strain XF56 with disruption in the *speC*, *speF* and *speB* genes responsible for putrescine biosynthesis. The cells without *gfpmut3* were included as negative controls, whereas the cells expressing *gfpmut3* from constitutive *lacIQ* promoter (*piq*) were shown as positive controls

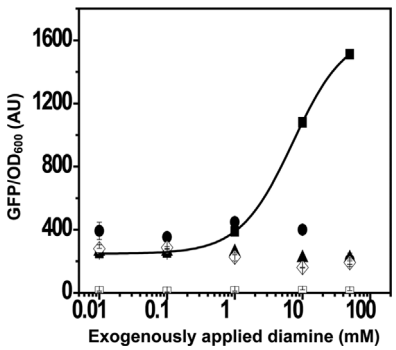


**FIGURE 3** Test of biosensor specificity. A typical biosensor strain *E. coli* MG1655 harboring plasmid pTacR2gfp was grown at 37°C in LB medium with exogenous addition of 1,3-diaminopropane (filled circle), putrescine (filled square), 1,5-diaminopentane (filled triangle) or 1,6-diaminohexane (open diamond). Cell fluorescence was measured at 20 hr upon diamine treatment. The cells without harboring pTacR2gfp were exposed to putrescine and included as negative control (open square). The best-fit curve of data to a Hill function was also shown

exposure in the LB medium, GFP fluorescence of the biosensor cells was indistinguishable from that of the control cells without putrescine treatment. After 6 hr, the fluorescence levels of the putrescine-treated cells were significantly higher than the untreated cells, which indicated the biosensor response in the LB medium. On the other hand, the putrescine-treated cells in M9 medium exhibited fluorescence at levels comparable with those of the untreated cells during the long time examined (24 hr), implying that the biosensor was not responsive to putrescine in the minimal medium. Interestingly, the responsiveness was restored when the biosensor was exposed to putrescine in the modified M9 medium, and the response time in this scenario was shortened to approximately 2 hr. Clearly, the existence or absence of ammonium in the glucose minimal medium dictated the responsive behavior of the putrescine biosensor cells. This might be due to that ammonium was a more favorable nitrogen source in minimal medium and its presence compromised uptake of the exogenously applied putrescine (see discussion below). Taken together, the results proved that it was possible to modulate biosensor response time by altering the culture medium.

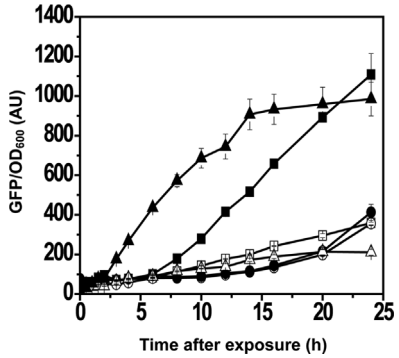


**FIGURE 4** Modulation of response kinetics for the putrescine biosensor. *E. coli* MG1655 harboring pTacR2gfp was exposed to 6 mM putrescine in LB medium (filled square), M9 medium (filled circle) or modified M9 medium with removal of ammonium chloride (filled triangle). Putrescine was added at time zero and fluorescence was monitored for the cells grown at 37°C for 24 hr. The cells grown with the same media without putrescine treatment were included as controls (open symbols)

## 3.5 | Modulation of response dynamics by altering PuuR level

As the expression level of PuuR would influence expression of the *gfpmut3* reporter, we next explored the effects of altering PuuR level on biosensing performance. Here, an alternative biosensor plasmid pS6 was constructed by inserting a *lacIQ* promoter-*puuR* expression cassette into the backbone of plasmid pTacR2gfp. Two additional biosensor strains were constructed by transforming plasmid pS6 into *E. coli* MG1655 and its *puuR* mutant (XF07C), and then exposed to putrescine in the LB medium or the modified M9 medium (Figure 5).

In both media, the two pS6-harboring strains exhibited lower basal expression of GFP, as compared with the former strain MG1655 (pTacR2gfp) with chromosomal *puuR* only. This result was expected because *puuR* expression from plasmid pS6 would give higher level of PuuR compared with its chromosomal expression only, and thus resulted in tighter repression on the *TacR2* promoter. In addition, the GFP expression levels upon induction with 100 mM putrescine in the LB medium were decreased by approximately 60% for the two pS6-harboring strains (Figure 5a). It was an extreme case that in the modified M9 medium, the expression of GFP was completely repressed in the pS6-harboring strains under the wide range of putrescine concentrations tested (Figure 5b). This unexpected phenomenon might be due to the retarded uptake of putrescine by the pS6-harboring strains because expression of the chromosomal *puuP* gene, which encodes the major putrescine transporter PuuP (Kurihara et al., 2005), was also repressed by PuuR. To explore the possibility, we monitored relative cell growth of the biosensor strains before and after putrescine exposure to the modified M9 medium, since uptake of extracellular putrescine was the only possible means to acquire nitrogen source for supporting cell growth. Indeed, almost no growth was observed for the strains harboring pS6, whereas the other strains showed appreciable cell growth under the experimental conditions (Supplementary Figure S2). Collectively, the results demonstrated that an alteration in PuuR expression modulated the response dynamics of the biosensor strains.

## 3.6 | Modulation of response dynamics by altering putrescine biosynthetic capability

Since *E. coli* was able to biosynthesize putrescine, the endogeneous levels of putrescine were postulated to affect response dynamics of the biosensor strains to external putrescine. In fact, wildtype *E. coli* MG1655 possessed two pathways for putrescine biosynthesis: one pathway generates putrescine through direct decarboxylation of ornithine catalyzed by either *speC* or *speF* gene product, whereas the other pathway involves arginine decarboxylation and agmatine

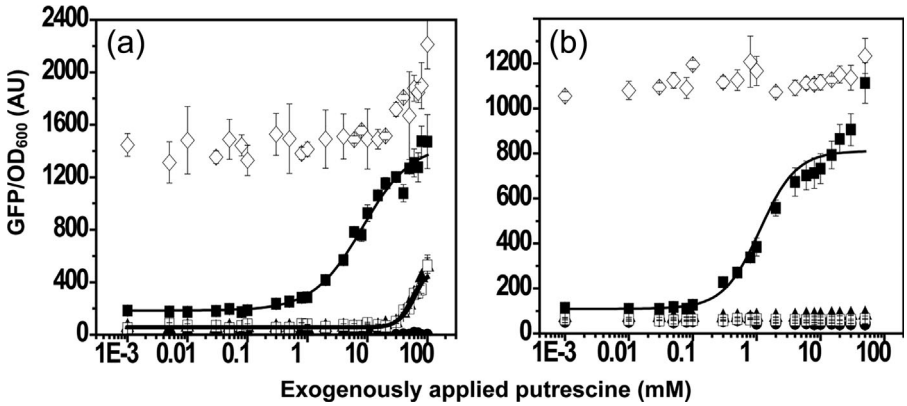


**FIGURE 5** Tuning detection range by varying PuuR levels in biosensors. The biosensor strains, with chromosome- or plasmid-based expression of PuuR, were exposed to various concentrations of putrescine in LB medium (**a**) and the modified M9 medium (**b**). Cell fluorescence was measured upon incubation at 37°C for 20 hr in the LB medium and for 10 hr in the modified M9 medium. The biosensor strains tested were *E. coli* MG1655 harboring pTacR2gfp (filled square), MG1655 harboring pS6 with plasmid-based PuuR coexpression (filled triangle), and XF07C harboring pS6 (open square). The PuuR deficient strain, *E. coli* XF07C harboring pTacR2gfp (open diamond) was included as positive control, whereas MG1655 without any plasmid was included as negative control (filled circle). The best-fit curves of data to a Hill function were also shown

hydrolysis by agmatinase (*speB*) to yield putrescine (Figure 1). As one can envision, chromosomal disruption of one or more putrescine biosynthetic genes would decrease the endogenous levels of putrescine in the engineered strains. Indeed, the endogenous level of putrescine was decreased by ~50% in the *speC* gene knockout strain (XF20C) and almost completely abolished in the *speC speB speF* triple knockout strain (XF56) when these strains were grown in glucose M9 medium (Supplementary Figure S1).

To explore response dynamics of the biosensors with attenuated putrescine biosynthetic capacity, we constructed another two biosensor strains by transforming plasmid pTacR2gfp into *E. coli* XF20C and XF56, respectively. These two strains, together with the prototype biosensor MG1655 (pTacR2gfp) as a control, were then exposed to 0–50 mM of putrescine in the modified M9 medium (Figure 6). It was observed that the response properties, including minimal and maximal expression levels, induction ratio and dose response curve changed dramatically. Notably, *E. coli* XF20C (pTacR2gfp) and XF56 (pTacR2gfp) responded more sensitively to exogenous putrescine, compared with the prototype biosensor. For example, the former two strains allowed a detection range of 0.04–0.65 mM and 0.03–0.72 mM, respectively, whereas the prototype biosensor gave a detection range of 0.11–7.18 mM (Supplementary Table S1). On the other hand, the dynamic range of *E. coli* XF20C (pTacR2gfp) and XF56 (pTacR2gfp) was decreased by approximately 22–25%. The above results clearly demonstrated that response dynamics of the putrescine biosensors could be modulated by altering putrescine biosynthetic capability of the chassis cells for biosensing. Nonetheless, the detailed mechanisms for the changes in biosensing properties remain unclear, which might be due to the fact that putrescine metabolism in *E. coli* was complicated by biosynthesis, degradation, utilization and uptake of extracellular putrescine.

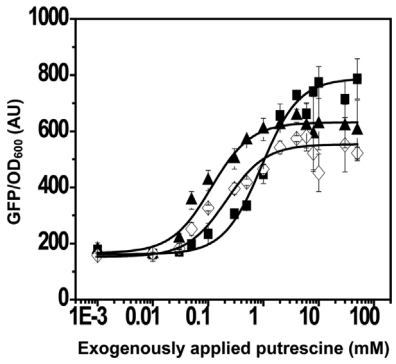


**FIGURE 6** Tuning detection range by modulating putrescine biosynthetic capability. The biosensor strains, with and without disruption in the chromosomal putrescine biosynthetic genes, harbored same reporting plasmid, pTacR2gfp. The recombinant strains were exposed to various concentrations of putrescine in the modified M9 medium and cell fluorescence was measured upon incubation at 37°C for 10 hr. Genetic backgrounds and symbols for the biosensors: MG1655 (wild-type, filled square), XF20C (*speC* mutant, filled triangle), and XF56 (*speC speB speF* triple mutant, open square)

## 3.7 | In vivo application of putrescine biosensors

Having demonstrated that response dynamics of the putrescine biosensors could be modulated by tuning biosensing components, we next explored utility of the engineered biosensors in vivo. First, we tested if the biosensors can report changes in intracellular putrescine levels caused by genetic modifications. To this end, the sensing plasmid pTacR2gfp was transformed into a series of *E. coli* strains with either attenuated or elevated putrescine biosynthetic capacity. Here, two putrescine overproduction strains ZQ19 and ZQ26 were included in the tests. Strain ZQ26 was also derived from *E. coli* MG1655 and constructed (see Supplementary Method for details) with chromosomally integrated expression cassette for an ornithine decarboxylase variant under the strong $P_{N25}$ promoter (Kammerer, Deuschle, Gentz, & Bujard, 1986). These recombinant strains were cultivated with glucose M9 medium to exponential growth phase with cell $OD_{600}$ at ~0.8–1.0. The bacterial cells were harvested and the endogenous levels of putrescine were quantified by HPLC. As expected, overexpression of the putrescine biosynthetic genes in strains ZQ26 and ZQ19 resulted in two- to threefold higher levels of intracellular putrescine, whereas the genetic backgrounds with deletion in one or more of the putrescine biosynthetic genes possessed decreased levels of endogenous putrescine (Figure 7). More interestingly, we observed a strong linear correlation ($R^2 = 0.9471$) between GFP output and endogenous putrescine levels that spanned a wide range of 0.048–18.049 mg g $DW^{-1}$. Hence, the above result demonstrated applicability of the putrescine biosensor for reporting putrescine biosynthetic capacity in *E. coli*.

Furthermore, we explored whether the putrescine biosensor could be utilized for reporting production titers in fermentation of putrescine overproduction strain. As a typical example, the putrescine overproducer *E. coli* ZQ19 (pTacR2gfp) was grown in baffled shake flask with minimal R/2 medium supplemented with 3 g $L^{-1}$ of $(NH_4)_2SO_4$ and 10 g $L^{-1}$ of glucose, which was developed as a production medium for C3 to C5 diamines in our previous studies (Chae, Kim, Choi, Park, & Lee, 2015; Park et al., 2012; Qian et al., 2009, 2011). As shown in Figure 8a, the extracellular titers of the strain increased slightly in the first 6 hr of cultivation and then sharply from 50 to 608.6 mg $L^{-1}$ in the following 6 hr of fermentation. It was very interesting that the cell fluorescence exhibited the same trend in changes as the excretory putrescine titer during the whole fermentation process. The results strongly suggested that there might be a link between cell fluorescence and excretory putrescine titer. Indeed, a correlation study identified an asymptotic model that well recapitulated the quantitative relationship between cell fluorescence intensity and excretory putrescine titer (Figure 8b). In addition, the asymptotic model was biotechnologically relevant with regard to the fermentation process. At the very beginning of fermentation, the extracellular putrescine level was negligible, whereas a basal level of endogenous putrescine was observed for the *E. coli* cells that had been grown overnight in LB medium. As the cells grew in the production medium, de novo putrescine biosynthesis resulted in elevated levels of endogenous putrescine leading to a gradual increase in cell

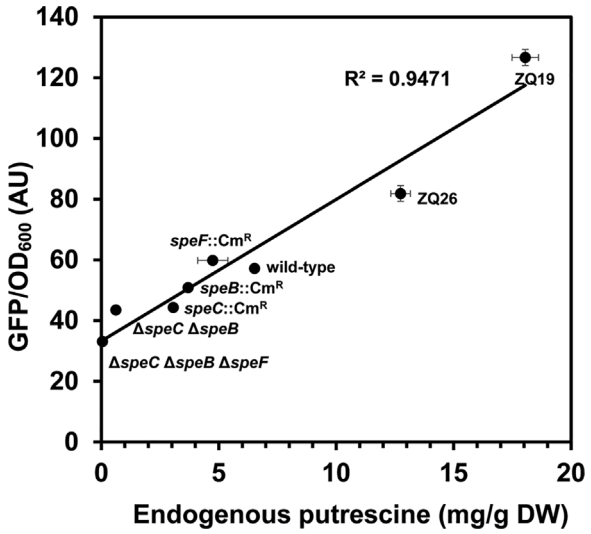


**FIGURE 7** Use of the biosensor for monitoring intracellular putrescine. The sensing plasmid pTacR2gfp was transformed into wild-type *E. coli* MG1655 and its derivatives with genetic knockouts or overexpressions of the putrescine biosynthetic genes. The recombinant strains were grown in M9 medium at 37°C. Fluorescence was measured when cell $OD_{600}$ reached 0.8 ~ 1.0, and intracellular putrescine levels were determined by HPLC as described in Methods section. Endogenous putrescine and normalized fluorescence intensities were plotted for each strain. A fitted straight line based on linear regression was shown

fluorescence, and the cell secreted putrescine into the extracellular medium to maintain putrescine homeostasis. At the later stage of fermentation, the endogenous putrescine was approaching an upper asymptote (saturation level) resulting in toxicity to the bacterial cells, whereas the putrescine titer increased gradually as long as there was sufficient glucose and ammonium in the culture medium that fueled putrescine biosynthesis and export. Overall, the results indicated that the putrescine biosensor was able to report putrescine production titers in shake flask fermentation.

## 4 | DISCUSSION

In this study, we report design, construction, and tuning of transcription factor-based biosensors for monitoring both exogenous and endogenous putrescine in *E. coli*. For initial biosensor design and construction, we sought to recruit the putrescine-responsive transcription factor PuuR and its naturally occurring cognate promoter. The *puuA* promoter appeared to be an attractive candidate for biosensor construction as the transcriptional level of *puuA* was induced approximately 13-fold in M9-tryptone medium supplemented with 0.2% putrescine (Kurihara et al., 2008). Unfortunately, the native *puuA* promoter was relatively weak in strength and insensitive to the absence of PuuR in our assay (Figure 2), which prevented its use as a reporting element and required the design of synthetic PuuR-responsive promoters. With the knowledge of PuuR-binding operator sequence in hand, which was a priori for any synthetic promoter design, we made a series of synthetic promoters by inserting one or more of the operator sequences into strong promoters in order to achieve desirable PuuR responsiveness and promoter activity. Notably, the spacer length between the −35 and −10 sequences of a prokaryotic promoter is one of important factors that affect the promoter strength. According to Aoyama et al. (1983), 17 bp-spacing appeared to be optimal for promoter activity in *E. coli* and was more frequently found in naturally occurring promoters than the spacing from 15 to 21 bp. Indeed, a rather low activity was observed for the native *puuA* promoter with a spacer length of 15 bp and a 20-bp PuuR binding sequence. Positioning of the 20 bp operator between the −35 and −10 regions of the two phage promoters did not result in active synthetic promoters, whereas such insertion in the *tac* promoter yielded the *TacR2* promoter with desirable activity and responsiveness (Figure 2). This result implied that in addition to spacer length, other promoter features might also play a role in determining promoter strength.

Response time is an important property of whole-cell biosensors for monitoring signals of interest (Liu, Evans, & Zhang, 2015). Our

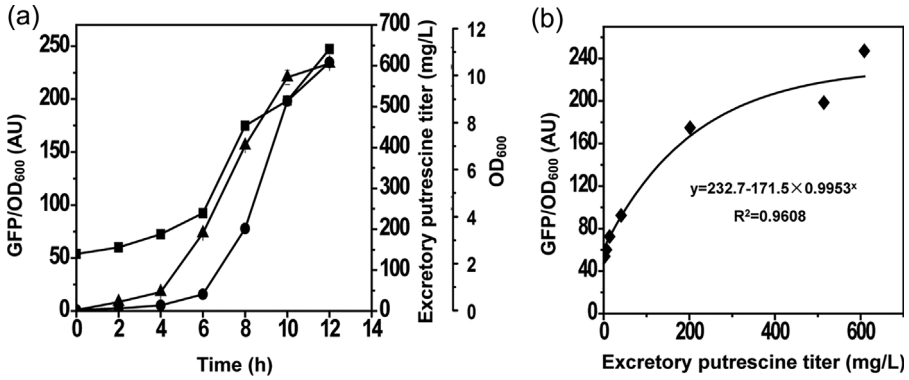


**FIGURE 8** Use of the biosensor for reporting extracellular production of putrescine. The engineered putrescine overproducer, *E. coli* ZQ19 was transformed with pTacR2gfp and grown in flasks with glucose R/2 medium at 37°C. (**a**) Cell fluorescence (filled square), $OD_{600}$ (filled triangle), and excretory putrescine titer (filled circle) were monitored for 12 hr. (**b**) Excretory putrescine titers and cell fluorescence were plotted. An asymptotic 1-D model was proposed to correlate cellular fluorescence and excretory putrescine titer, which quantitatively described the dynamic changes of intracellular and extracellular putrescine levels for the engineered cell factory

results demonstrated that it was possible to modulate response time of the putrescine whole-cell biosensors by altering culture medium. It was surprising that the response time upon putrescine exposure was shortened from 6 hr in rich medium to 2 hr in modified glucose M9 medium, whereas the presence of ammonium in the original M9 medium abolished the responsiveness (Figure 4). According to Reitzer (2003), *E. coli* can assimilate both inorganic (ammonia) and organic nitrogen sources (e.g., amino acids or putrescine), and ammonia supports the fastest growth rate and is therefore considered the preferred nitrogen source. From a metabolic point of view, ammonia is directly assimilated in reactions catalyzed by glutamate dehydrogenase or glutamine synthetase. However, when putrescine is utilized as a sole nitrogen source in glucose minimal medium, putrescine is first transported inside cells of *E. coli* via importer PuuP and then catabolized to release the direct nitrogen source, ammonia (Kurihara et al., 2008). Therefore, it was reasonable that ammonia was preferentially assimilated in the medium containing both ammonia and putrescine, and the existence of abundant ammonia might compromise import of putrescine into the bacterial cells for triggering responses. One can envision that the uptake of extracellular target chemical by a whole-cell biosensor is the first step for triggering output events and could be a critical factor in determining response time of the biosensor. Therefore, whether nutrients in the culture medium affect import of the chemical to be monitored should be taken into consideration in biosensing studies, even though this issue has often been ignored in previous studies.

We also explored tuning of the putrescine biosensors and found that many properties of the repressor-based biosensors can be modulated by different strategies. In addition to the strategy of designing synthetic PuuR-responsive promoter, we also tested the effects of varying PuuR expression and altering endogenous putrescine biosynthetic capacity by chromosomal modifications. The strategy of expression level control on PuuR was able to modulate background output and biosensor dynamic range, which coincided with previous studies in tuning other transcriptional repressor-based biosensors (Liu, Xiao, Evans, & Zhang, 2015; Merulla & van der Meer, 2016). More importantly, we demonstrated a previously unknown strategy of modulating endogenous biosynthetic capacity for modulating biosensor dynamics (Figure 6). It was notable that a reduction in endogenous putrescine biosynthetic capacity could improve sensitivity of the biosensor to external putrescine. This effect was possibly due to autonomous overexpression of the chromosomal *puuP* gene encoding the main putrescine importer in minimal medium, which facilitated uptake of extracellular putrescine even at very low concentrations and resulted in higher biosensor sensitivity. Therefore, the endogenous biosynthetic capacity of chassis cells should be taken into consideration for constructing and tuning other whole-cell biosensors. To further optimize biosensor performance, other strategies may also be considered including mutagenesis of the synthetic PuuR-responsive promoter to tune promoter strength and PuuR affinity or the modification of PuuR effector binding domain to modulate its affinity with putrescine. Since the identification of design constraints in transcription-factor-based biosensors (Mannan et al., 2017), the above strategies may be examined in combination to achieve orthogonal control and optimization of diverse biosensor parameters.

Finally, we have demonstrated the capacity of the biosensor to report different levels of endogenous putrescine in *E. coli* variants as well as excretory production titers of putrescine from an overproducing strain. The PuuR-based biosensor system can potentially enable fast bioprocess optimization by online reporting putrescine titers and high-throughput screening of *E. coli* mutants with improved biosynthetic capacity through random genome engineering. At last, the overall strategies described here for tuning biosensors should be useful for future design and engineering of repressor-based biosensors for monitoring other highly regulated cell metabolites in prokaryotes.

## ACKNOWLEDGMENTS

We thank D.E. Cameron for providing us with plasmids pZA2pBAD-gfp and pECA102. The program for professor of special appointment (Eastern Scholar) at Shanghai Institutions of Higher Learning is appreciated. SYL acknowledges the support from KAIST-Hanwha Chemical Future Technology Institute. The authors have filed a patent related to this work.

## ORCID

*Sang Yup Lee* http://orcid.org/0000-0003-0599-3091
*Zhi-Gang Qian* http://orcid.org/0000-0002-0133-0605

## REFERENCES

Ang, J., Harris, E., Hussey, B. J., Kil, R., & McMillen, D. R. (2013). Tuning response curves for synthetic biology. *ACS Synthetic Biology*, *2*(10), 547–567.

Aoyama, T., Takanami, M., Ohtsuka, E., Taniyama, Y., Marumoto, R., Sato, H., & Ikehara, M. (1983). Essential structure of *E. coli* promoter: Effect of spacer length between the two consensus sequences on promoter function. *Nucleic Acids Research*, *11*(17), 5855–5864.

Bereza-Malcolm, L. T., Mann, G., & Franks, A. E. (2015). Environmental sensing of heavy metals through whole cell microbial biosensors: A synthetic biology approach. *ACS Synthetic Biology*, *4*(5), 535–546.

Cerminati, S., Soncini, F. C., & Checa, S. K. (2011). Selective detection of gold using genetically engineered bacterial reporters. *Biotechnology and Bioengineering*, *108*(11), 2553–2560.

Chae, T. U., Kim, W. J., Choi, S., Park, S. J., & Lee, S. Y. (2015). Metabolic engineering of *Escherichia coli* for the production of 1,3-diaminopropane, a three carbon diamine. *Scientific Reports*, *5*, 13040.

Chong, H., & Ching, C. B. (2016). Development of colorimetric-based whole-cell biosensor for organophosphorus compounds by engineering transcription regulator DmpR. *ACS Synthetic Biology*, *5*(11), 1290–1298.

Chou, H. H., & Keasling, J. D. (2013). Programming adaptive control to evolve increased metabolite production. *Nature Communications*, *4*, 2595.

Dahl, R. H., Zhang, F., Alonso-Gutierrez, J., Baidoo, E., Batth, T. S., Redding-Johanson, A. M., … Keasling, J. D. (2013). Engineering dynamic pathway regulation using stress-response promoters. *Nature Biotechnology*, *31*(11), 1039–1046.

Dietrich, J. A., Shis, D. L., Alikhani, A., & Keasling, J. D. (2013). Transcription factor-based screens and synthetic selections for microbial small-molecule biosynthesis. *ACS Synthetic Biology*, *2*(1), 47–58.

Fang, M., Wang, T., Zhang, C., Bai, J., Zheng, X., Zhao, X., … Xing, X. H. (2016). Intermediate-sensor assisted push-pull strategy and its application in heterologous deoxyviolacein production in *Escherichia coli*. *Metabolic Engineering*, *33*, 41–51.

Jang, Y. S., Kim, B., Shin, J. H., Choi, Y. J., Choi, S., Song, C. W., … Lee, S. Y. (2012). Bio-based production of C2-C6 platform chemicals. *Biotechnology and Bioengineering*, *109*(10), 2437–2459.

Kammerer, W., Deuschle, U., Gentz, R., & Bujard, H. (1986). Functional dissection of *Escherichia coli* promoters: Information in the transcribed region is involved in late steps of the overall process. *EMBO Journal*, *5*(11), 2995–3000.

Kurihara, S., Oda, S., Kato, K., Kim, H. G., Koyanagi, T., Kumagai, H., & Suzuki, H. (2005). A novel putrescine utilization pathway involves gamma-glutamylated intermediates of *Escherichia coli* K-12. *The Journal of Biological Chemistry*, *280*(6), 4602–4608.

Kurihara, S., Oda, S., Tsuboi, Y., Kim, H. G., Oshida, M., Kumagai, H., & Suzuki, H. (2008). γ-Glutamylputrescine synthetase in the putrescine utilization pathway of *Escherichia coli* K-12. *The Journal of Biological Chemistry*, *283*(29), 19981–19990.

Li, Y. F., & Yu, Z. (2015). Construction and evaluation of a genetic construct for specific detection and measurement of propionate by whole-cell bacteria. *Biotechnology and Bioengineering*, *112*(2), 280–287.

Libis, V., Delépine, B., & Faulon, J. L. (2016). Expanding biosensing abilities through computer-aided design of metabolic pathways. *ACS Synthetic Biology*, *5*(10), 1076–1085.

Liu, D., Evans, T., & Zhang, F. (2015). Applications and advances of metabolite biosensors for metabolic engineering. *Metabolic Engineering*, *31*, 35–43.

Liu, D., Xiao, Y., Evans, B. S., & Zhang, F. (2015). Negative feedback regulation of fatty acid production based on a malonyl-CoA sensor-actuator. *ACS Synthetic Biology*, *4*(2), 132–140.

Lutz, R., & Bujard, H. (1997). Independent and tight regulation of transcriptional units in *Escherichia coli* via the LacR/O, the TetR/O and AraC/I1-I2 regulatory elements. *Nucleic Acids Research*, *25*(6), 1203–1210.

Mahr, R., & Frunzke, J. (2016). Transcription factor-based biosensors in biotechnology: Current state and future prospects. *Applied Microbiology and Biotechnology*, *100*(1), 79–90.

Mannan, A. A., Liu, D., Zhang, F., & Oyarzún, D. A. (2017). Fundamental design principles for transcription-factor-based metabolite biosensors. *ACS Synthetic Biology*, *6*(10), 1851–1859.

Merulla, D., & van der Meer, J. R. (2016). Regulatable and modulable background expression control in prokaryotic synthetic circuits by auxiliary repressor binding sites. *ACS Synthetic Biology*, *5*(1), 36–45.

Moser, F., Horwitz, A., Chen, J., Lim, W., & Voigt, C. A. (2013). Genetic sensor for strong methylating compounds. *ACS Synthetic Biology*, *2*(10), 614–624.

Mustafi, N., Grünberger, A., Kohlheyer, D., Bott, M., & Frunzke, J. (2012). The development and application of a single-cell biosensor for the detection of L-methionine and branched-chain amino acids. *Metabolic Engineering*, *14*(4), 449–457.

Nemoto, N., Kurihara, S., Kitahara, Y., Asada, K., Kato, K., & Suzuki, H. (2012). Mechanism for regulation of the putrescine utilization pathway by the transcription factor PuuR in *Escherichia coli* K-12. *Journal of Bacteriology*, *194*(13), 3437–3447.

Nguyen, A. Q., Schneider, J., Reddy, G. K., & Wendisch, V. F. (2015). Fermentative production of the diamine putrescine: System metabolic engineering of *Corynebacterium glutamicum*. *Metabolites*, *5*(2), 211–231.

Park, J. M., Park, H. M., Kim, W. J., Kim, H. U., Kim, T. Y., & Lee, S. Y. (2012). Flux variability scanning based on enforced objective flux for identifying gene amplification targets. *BMC Systems Biology*, *6*, 106.

Qian, Z. G., Xia, X. X., & Lee, S. Y. (2009). Metabolic engineering of *Escherichia coli* for the production of putrescine: A four carbon diamine. *Biotechnology and Bioengineering*, *104*(4), 651–662.

Qian, Z. G., Xia, X. X., & Lee, S. Y. (2011). Metabolic engineering of *Escherichia coli* for the production of cadaverine: A five carbon diamine. *Biotechnology and Bioengineering*, *108*(1), 93–103.

Raman, S., Rogers, J. K., Taylor, N. D., & Church, G. M. (2014). Evolution-guided optimization of biosynthetic pathways. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(50), 17803–17808.

Ramos, J. L., Martínez-Bueno, M., Molina-Henares, A. J., Terán, W., Watanabe, K., Zhang, X., … Tobes, R. (2005). The TetR family of transcriptional repressors. *Microbiology and Molecular Biology Reviews*, *69*(2), 326–356.

Reitzer, L. (2003). Nitrogen assimilation and global regulation in *Escherichia coli*. *Annual Review of Microbiology*, *57*, 155–176.

Rogers, J. K., & Church, G. M. (2016). Genetically encoded sensors enable real-time observation of metabolite production. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(9), 2388–2393.

Roth, A., & Breaker, R. R. (2009). The structural and functional diversity of metabolite-binding riboswitches. *Annual Review of Biochemistry*, *78*, 305–334.

Schendzielorz, G., Dippong, M., Grünberger, A., Kohlheyer, D., Yoshida, A., Binder, S., … Eggeling, L. (2014). Taking control over control: Use of product sensing in single cells to remove flux control at key enzymes in biosynthesis pathways. *ACS Synthetic Biology*, *3*(1), 21–29.

Siedler, S., Stahlhut, S. G., Malla, S., Maury, J., & Neves, A. R. (2014). Novel biosensors based on flavonoid-responsive transcriptional regulators introduced into *Escherichia coli*. *Metabolic Engineering*, *21*, 2–8.

Skjoedt, M. L., Snoek, T., Kildegaard, K. R., Arsovska, D., Eichenberger, M., Goedecke, T. J., … Keasling, J. D. (2016). Engineering prokaryotic transcriptional activators as metabolite biosensors in yeast. *Nature Chemical Biology*, *12*(11), 951–958.

Smanski, M. J., Zhou, H., Claesen, J., Shen, B., Fischbach, M. A., & Voigt, C. A. (2016). Synthetic biology to access and expand nature's chemical diversity. *Nature Reviews Microbiology*, *14*(3), 135–149.

Stanton, B. C., Nielsen, A. A., Tamsir, A., Clancy, K., Peterson, T., & Voigt, C. A. (2014). Genomic mining of prokaryotic repressors for orthogonal logic gates. *Nature Chemical Biology*, *10*(2), 99–105.

Tabor, C. W., & Tabor, H. (1985). Polyamines in microorganisms. *Microbiological Reviews*, *49*(1), 81–99.

Tang, S. Y., & Cirino, P. C. (2011). Design and application of a mevalonate-responsive regulatory protein. *Angewandte Chemie International Edition in English*, *50*(5), 1084–1086.

Tang, S. Y., Fazelinia, H., & Cirino, P. C. (2008). AraC regulatory protein mutants with altered effector specificity. *Journal of the American Chemical Society*, *130*(15), 5267–5271.

Tang, S. Y., Qian, S., Akinterinwa, O., Frei, C. S., Gredell, J. A., & Cirino, P. C. (2013). Screening for enhanced triacetic acid lactone production by recombinant *Escherichia coli* expressing a designed triacetic acid lactone reporter. *Journal of the American Chemical Society*, *135*(27), 10099–10103.

Wang, M., Li, S., & Zhao, H. (2016). Design and engineering of intracellular-metabolite-sensing/regulation gene circuits in *Saccharomyces cerevisiae*. *Biotechnology and Bioengineering*, *113*(1), 206–215.

Xiao, Y., Bowen, C. H., Liu, D., & Zhang, F. (2016). Exploiting nongenetic cell-to-cell variation for enhanced biosynthesis. *Nature Chemical Biology*, *12*(5), 339–344.

Xu, P., Li, L., Zhang, F., Stephanopoulos, G., & Koffas, M. (2014). Improving fatty acids production by engineering dynamic pathway regulation and metabolic control. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(31), 11299–11304.

Yang, J., Seo, S. W., Jang, S., Shin, S. I., Lim, C. H., Roh, T. Y., & Jung, G. Y. (2013). Synthetic RNA devices to expedite the evolution of metabolite-producing microbes. *Nature Communications*, *4*, 1413.

Zhang, F., Carothers, J. M., & Keasling, J. D. (2012). Design of a dynamic sensor-regulator system for production of chemicals and fuels derived from fatty acids. *Nature Biotechnology*, *30*(4), 354–359.

Zhang, J., Barajas, J. F., Burdu, M., Ruegg, T. L., Dias, B., & Keasling, J. D. (2017). Development of a transcription factor-based lactam biosensor. *ACS Synthetic Biology*, *6*(3), 439–445.

Zhang, J., Jensen, M. K., & Keasling, J. D. (2015). Development of biosensors and their application in metabolic engineering. *Current Opinion in Chemical Biology*, *28*, 1–8.

Zhang, J., Sonnenschein, N., Pihl, T. P., Pedersen, K. R., Jensen, M. K., & Keasling, J. D. (2016). Engineering an NADPH/$NADP^+$ redox biosensor in yeast. *ACS Synthetic Biology*, *5*(12), 1546–1556.

Zhou, L. B., & Zeng, A. P. (2015). Exploring lysine riboswitch for metabolic flux control and improvement of L-lysine synthesis in *Corynebacterium glutamicum*. *ACS Synthetic Biology*, *4*(6), 729–734.

## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

**How to cite this article:** Chen X-F, Xia X-X, Lee SY, Qian Z-G. Engineering tunable biosensors for monitoring putrescine in *Escherichia coli*. *Biotechnology and Bioengineering*. 2018;115:1014–1027. https://doi.org/10.1002/bit.26521