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Isoleucyl-tRNA synthetase mutant based whole-cell biosensor for high-throughput selection of isoleucine overproducers

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ABSTRACT

Whole-cell amino acid biosensors can sense the concentrations of certain amino acids and output easily detectable signals, which are important for construction of microbial producers. However, many reported biosensors have poor specificity because they also sense non-target amino acids. Besides, biosensors for many amino acids are still unavailable. In this study, we proposed a new strategy for constructing whole-cell biosensors based on aminoacyl-tRNA synthetases (aaRSs), which take the advantage of their universality and intrinsically specific binding ability to corresponding amino acids. Taking isoleucine biosensor as an example, we first mutated the isoleucyl-tRNA synthetase in *Escherichia coli* to dramatically decrease its affinity to isoleucine. The engineered cells specifically sensed isoleucine and output isoleucine dose-dependent cell growth as an easily detectable signal. To further expand the sensing range, an isoleucine exporter was overexpressed to enhance excretion of intracellular isoleucine. Since cells equipped with the optimized whole-cell biosensor showed accelerated growth when cells produced higher concentrations of isoleucine, the biosensor was successfully applied in high-throughput selection of isoleucine overproducers from random mutation libraries. This work demonstrates the feasibility of engineering aaRSs to construct a new kind of whole-cell biosensors for amino acids. Considering all twenty proteinogenic and many non-canonical amino acids have their specific aaRSs, this strategy should be useful for developing biosensors for various amino acids.

1. Introduction

Amino acids have wide applications as food additives, animal feed, pharmaceuticals, cosmetics, polymer materials, etc. Because of the irreplaceable function of amino acids and their derivatives and the continuous development of their novel uses, the market is growing rapidly with a predicted annual increase of 6–8% (Tonouchi and Ito 2017). Most commercialized amino acids are produced by large-scale fermentation using microbes with systematic and global genetic modifications (Leuchtenberger et al., 2005). The powerful multiplex and automated genome engineering technologies (Wang et al. 2009, 2018b) and random mutagenesis technologies (Han et al., 2020) allow simultaneously introducing genetic modifications at multiple genomic targets. However, the mutation libraries generated by these methods usually bear great diversity, which requires labor-intensive and time-consuming evaluation via fermentation and subsequent product analysis to finally obtain the desired overproducers. This seriously affects the efficiency of strain development.

Whole-cell amino acid biosensors can transmit the concentration information of amino acid into easily detectable signals, such as fluorescence, antibiotic resistance, and growth phenotype. They allow real-time and *in-situ* monitoring of amino acid products, and therefore can be developed as high-throughput selection tools for strain engineering. Several amino acid biosensors have been successfully developed by using different genetic sensing devices including transcription factors, riboswitches, and fluorescence resonance energy transfer systems (Binder et al., 2012; Choi et al., 2013; Liu et al., 2015; Mohsin et al., 2013; Mohsin and Ahmad 2014; Niazi et al., 2008; Wang et al., 2016;

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Yang et al., 2013). For example, in order to screen lysine overproducers from random mutants of *Corynebacterium glutamicum*, a biosensor composing of a lysine sensitive transcription factor LysG and a regulated fluorescence gene was constructed (Binder et al., 2012). However, LysG responds not only to lysine but also to arginine and histidine. The poor specificity is commonly observed for transcription factor-based biosensors, which increases the number of false positives during screening. In another example, a lysine sensitive aspartate kinase III gene riboswitch linked with a tetracycline resistant gene was applied to evolve a chimeric aspartate kinase (Wang et al., 2015). However, the values of the apparent dissociation constant of riboswitches are usually much lower (e.g. ~0.15 mg/L lysine for aspartate kinase III gene riboswitch) than the intracellular amino acid concentrations (e.g. >50 mg/L of lysine in wild-type *Escherichia coli*) (Guo et al., 2013; Sudarsan et al., 2003). Therefore, the over-occupation of riboswitches at low effector concentrations seriously limits the application in screening high-level amino acid producers. In addition, suitable sensing devices for many amino acids have not been discovered yet. Therefore, universal strategies of establishing biosensors for specifically sensing an amino acid are urgently desired.

Aminoacyl-tRNA synthetases (aaRSs) help twenty proteinogenic amino acids be accurately incorporated to synthesize proteins. Each aaRS specifically recognizes its cognate amino acid and the corresponding tRNA, and then produces aminoacyl-tRNA by a two-step catalytic mechanism (Fersht and Kaethner 1976; Perona and Hadd 2012). The size of the binding pocket, formation of multiple isoelectric interactions between amino acid and aaRS, and induced-fit mechanism between amino acid, aaRS, and tRNA constitute the triple insurance for the accurate synthesis of aminoacyl-tRNA (Perona and Hadd 2012; Rodgers and Shiozawa 2008). Mutations at the amino acid binding sites of aaRS were reported to decrease the binding affinity to cognate amino acid and consequently hindered protein synthesis and cell growth (Clarke et al., 1988; Iaccarino and Berg 1971).

In this study, we demonstrated that engineered aaRSs could serve as promising sensing devices for constructing amino acid biosensors because of their universality and inherent specificity. As a proof-of-concept, we employed an *E. coli* isoleucyl-tRNA synthetase (IleRS) mutant with ~11000-fold decreased binding affinity compared with the wild-type IleRS to develop a growth-coupled whole-cell isoleucine biosensor. To expand the isoleucine sensing range, an isoleucine exporter was additionally overexpressed. Cells equipped with the biosensor showed growth defect but isoleucine producing cells showed growth recovery due to increased intracellular isoleucine concentration. Based on this observation, we applied the biosensor to select isoleucine overproducers from random mutation libraries. This study provides a promising strategy for developing whole-cell biosensors for all twenty proteinogenic and possibly non-canonical amino acids by using a proper mutant aaRS.

2. Material and methods

2.1. Enzymes and chemicals

The Phusion High-Fidelity DNA polymerase was purchased from Thermo Fisher Scientific (Massachusetts, USA). The DNA Purification Kit was obtained from TIANGEN (Beijing, China). The CloneExpress II/MultiS One Step Cloning Kit was purchased from Vazyme (Nanjing, China). The chemically competent *E. coli* strain DH5α and the pEASY-Blunt Cloning Kit were purchased from Transgene (Beijing, China). Oligonucleotides were synthesized by GENEWIZ (Beijing, China). Isoleucine, amino acids, and antibiotics were purchased from Solarbio (Beijing, China). Other chemicals used in this study are of analytical grade or better.

2.2. Cultivation media

The Luria-Bertani (LB) medium contains 5 g/L yeast extract, 10 g/L tryptone, and 10 g/L sodium chloride. The LBI medium contains additional 1.6 g/L isoleucine in the LB medium. The test medium (TM) contains 30 g/L glucose, 2 g/L yeast extract, 5 g/L $(NH_4)_2SO_4$, 1 g/L citric acid, 2 g/L KH_2PO_4, 0.7 g/L $MgSO_4{\cdot}7H_2O$, 0.05 g/L $FeSO_4{\cdot}7H_2O$, 0.05 g/L $MnSO_4{\cdot}H_2O$, and B group vitamins (V_{B1},V_{B3},V_{B5},V_{B7}, and V_{B12} with each at 0.8 mg/L). The pH of all the media was adjusted to 7.0. Agar plates were prepared by adding 2% agar powder to liquid media. Without special description, ampicillin at 100 mg/L and/or kanamycin at 25 mg/L were routinely added according to the resistance of strains. For plasmid and strain construction, cells were cultivated at 37 °C in flasks shaking at 200 rpm. For growth test, iterative transfer, and fermentation test, cells were cultivated at 37 °C in 24-deep-well plates with each well containing 1 mL medium, shaking at 800 rpm in INFORS Microtron (INFORS HT Multitron Pro, Switzerland).

2.3. Plasmid construction

The main bacterial strains, plasmids, and primers used in this study are listed in Table S1. *E. coli* strain DH5α was used for the plasmid construction and cultivated routinely using LB medium. For overexpressing threonine dehydratase IlvA, the plasmid pSB backbone, P_{trc} promoter, and the wild-type *ilvA* gene were amplified using primer pairs pSB-F/R, trc-F/R, and ilvA1/2, from plasmid pSB (Wang et al., 2019), plasmid pTrc99A, and *E. coli* MG1655 genomic DNA, respectively. The three DNA fragments were ligated using the CloneExpress MultiS One Step Cloning Kit to generate plasmid pSB-*ilvA*. Four *ilvA* mutations (C1339T, G1341T, C1351G, T1352C) that can release isoleucine feedback inhibition (Park et al., 2012) were introduced using a site-directed mutagenesis method (Liu and Naismith 2008) with two rounds of PCR and primer pairs ilvA3/4 and ilvA5/6, generating plasmid pSB-$ilvA^{M}$.

For overexpressing isoleucine exporter YgaZH, a basic plasmid pTrcD was first constructed by PCR amplification of the pTrc99A backbone with primer pair pTrc-F/R, and subsequent self-ligation. The pTrcD backbone was then amplified with primer pair pTrcD-F/R, and *ygaZH* was amplified from *E. coli* MG1655 genomic DNA with primer pair ygaZH-F/R. The two PCR fragments were ligated to generate plasmid pTrcD-*ygaZH*. Then, constitutive promoters P_{J23116}, P_{J23106}, and P_{J23100} were individually introduced into pTrcD-*ygaZH* to control *ygaZH* expression by PCR using primer pairs 116F/R, 106F/R, and 100F/R and subsequent self-ligation, generating plasmids pTrcD-P_{J23116}-*ygaZH*, pTrcD-P_{J23106}-*ygaZH*, and pTrcD-P_{J23100}-*ygaZH*, respectively.

2.4. Strain construction

To introduce base mutations (G280A and C282G, corresponding to the amino acid substitution G94R) (Clarke et al., 1988) into the original *ileRS* gene of *E. coli* MG1655 chromosome, a two-step λ-red recombination method was used as described previously (Zhang et al., 2007). First, the fragment containing the *ileRS* gene, the 500-bp upstream, and 500-bp downstream of *ileRS* was amplified from MG1655 genomic DNA using primer pair S–F/R. The fragment was ligated with plasmid pEASY-blunt, resulting in plasmid pEA-up-*ileRS*-down. The G280A and C282G mutations were introduced into pEA-up-*ileRS*-down by PCR-based site-directed mutagenesis method (Liu and Naismith 2008) with primer pair S1/2, to generate plasmid pEA-up-$ileRS^{G94R}$-down. The *cat-sacB* cassette encoding chloramphenicol acetyltransferase and levansucrase was amplified from plasmid pSC3 (Wang et al., 2018a) using primer pair sacB-F/R, and pEA-up-$ileRS^{G94R}$-down backbone was amplified using primer pair blunt-F/R. These two PCR fragments were ligated to generate plasmid pEA-up-*cat-sacB-*$ileRS^{G94R}$-down. Next, the fragments for the first and second rounds of recombination were amplified from pEA-up-*cat-sacB-*$ileRS^{G94R}$-down and pEA-up-$ileRS^{G94R}$-down, respectively, using primer pair S–F/R. Finally, the

recombinant fragments were used to perform the two-step λ-red recombination process to introduce the mutations of *ileRS* into the chromosome. The generated strain MG1655 (*ileRS*^{G94R}) was named as MG1655M. Replacement of the native promoter of *ilvEDA* (Park et al., 2012) on the MG1655M chromosome with the P_{trc} promoter was carried out by using the same method described above, producing strain MG1655 (*ileRS*^{G94R}, P_{ilvEDA}:P_{trc}).

The plasmids pTrcD, pTrcD-P_{J23116}-*ygaZH*, pTrcD-P_{J23106}-*ygaZH*, and pTrcD-P_{J23100}-*ygaZH* were individually transferred into MG1655M via electro-transformation, resulting in strains MG1655MYC, MG1655MY1, MG1655MY2, and MG1655MY3, respectively. The plasmids pSB, pSB-*ilvA*, and pSB-*ilvA*^{M} were introduced into MG1655MY3 to generate strains Ile0, Ile1, and Ile2, respectively. The plasmids pTrcD-P_{J23100}-*ygaZH* and pSB-*ilvA*^{M} were co-transformed into strain MG1655 (*ileRS*^{G94R}, P_{ilvEDA}:P_{trc}) to generate strain Ile3.

2.5. Growth assay

Cells were cultivated overnight in LBI medium. The cultures were centrifuged and resuspended in 10-fold volume of TM medium. After further incubation in TM for 2 h, the cultures were used as seeds to inoculate fresh TM medium with an initial optical density at 600 nm (OD_{600}) of 0.1. For testing cell growth with addition of different amino acids, the amino acids were individually added to a final concentration of 0.5 g/L. To test the response of cell growth to isoleucine supplement, isoleucine was added to different final concentrations. To monitor cell growth, 100 μL culture was taken periodically into a Greiner 96-well microplate, diluted with TM to a final volume of 200 μL. OD_{600} was measured using a microplate reader (SpectraMax 190 from Molecular Devices, USA).

Growth of strains Ile0, Ile1, Ile2, and Ile3 on TM agar plates were tested. The seeds preparation process was the same as that for testing cell growth in liquid TM medium. The seeds were diluted by 10^6 times with TM medium, and 100 μL of each diluent was spread over the agar plate and cultivated at 37 °C for 30 h. To determine the colony size, the colony images were captured with Tanon 1600 Gel Image System (Tanon, China), and colony sizes were analyzed using the Tanon Colon software according to the colony pixels (Saleski et al., 2019).

2.6. Selection of Ile1 from a mixed culture of Ile1 and Ile0

Strains Ile1 and Ile0 were individually cultivated overnight in 50 mL LBI medium in flasks, washed once, and resuspended in 50 mL TM. Then, Ile1 and Ile0 cells were mixed at a ratio of 1:10^4. The mixture was diluted by 10^2 times, and spread over the TM and the LBI agar plates, respectively. The plates were incubated at 37 °C for 30 h until visible colonies appeared on the TM plates. Ten large colonies on the TM agar plates were picked and plasmids were extracted and verified by sequencing using the primer pilvA-T.

2.7. Selection of isoleucine overproducers from randomly mutated libraries

Strain MG1655M was used as the starting strain for random mutagenesis and selection of isoleucine producers. After cultivation in LBI medium to an OD_{600} of 1.0, 1 mL culture of MG1655M was centrifuged, washed once, and resuspended in 10 mL phosphate buffer (pH 7.4). The suspension was subjected to UV-light treatment for 30 s (~70% lethal rate according to a pre-experiment). Eight parallel samples of the UV-treated suspension (~10^5 survivals for each sample) were cultured in flasks containing 50 mL LBI medium in dark to an OD_{600} of ~0.6. The cultures were prepared as competent cells, transferred with pTrcD-P_{J23100}-*ygaZH*, further cultured in LBI medium for 2 h, and spread over the LBI agar plates with 100 mg/L ampicillin. For each sample, ~10^6 transformants were scraped from the agar plates and suspended in TM medium. Afterwards, eight samples were individually inoculated into TM medium in a 24-deep-well plate with an initial OD_{600} of 0.1. After cultivated for 8 h, the cultures were iteratively transferred in TM medium with an inoculation ratio of 0.2% (v/v). Cells from the starting culture, the 5th transfer, and the 10th transfer were spread over LBI agar plates with 100 mg/L ampicillin. After cultivation at 37 °C overnight, ten colonies were randomly picked from each plate and their isoleucine production capacities were tested.

2.8. Isoleucine production test

To determine the isoleucine production capacity of selected colonies, the seeds were prepared in the same way as described above for determination of cell growth. The seeds were then inoculated into TM medium in a 24-deep-well plate with an initial OD_{600} of 0.1. The cells were cultivated for 24 h, and the extracellular isoleucine concentration was determined using HPLC.

2.9. Quantification of extracellular and intracellular isoleucine concentration

To quantify the extracellular isoleucine concentration, the cell cultures were centrifuged at 12,000 rpm for 5 min, and isoleucine in supernatant was analyzed after 2,4-fluoro-dinitrobenzene precolumn derivatization using HPLC equipped with a UV detector (Shimadzu, Japan) and an Agilent HC-C18 column (4.6 × 250 mm) according to a previously described protocol (Gioia et al., 2007). The concentration of isoleucine was calculated through a calibration curve obtained with the standard isoleucine solution. To quantify the intracellular isoleucine concentration, cells were separated by a silicone oil centrifugation method (Forget et al., 1993). The cell pellets were resuspended in phosphate buffer (pH 7.4) to an OD_{600} of 60. Mechanical lysis of cells was performed with glass beads using the FastPrep-24 Classic Instrument (MP Biomedicals, USA). The cellular debris was removed by centrifugation at 12,000 rpm for 10 min at 4 °C, and the supernatant was used for HPLC analysis. The corresponding cellular aqueous volume was calculated according to a former report (Bennett et al., 2008). The intracellular isoleucine concentration was calculated according to the corresponding intracellular isoleucine weight and cell aqueous volume.

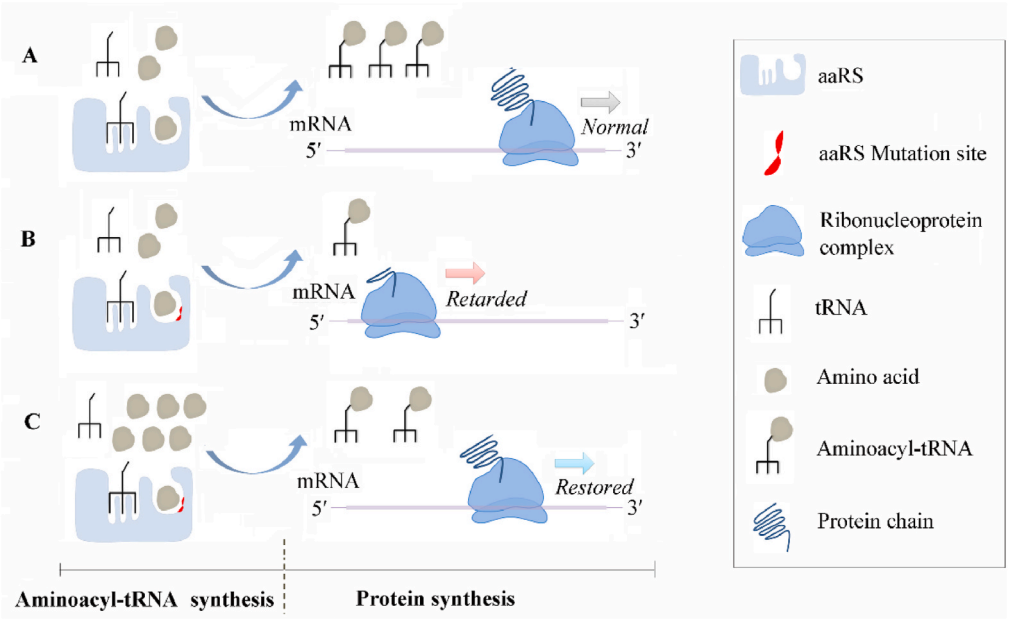
3. Results

3.1. Design of aaRS-based amino acid biosensors

Since an aaRS binds its cognate amino acid and the corresponding tRNA with two separate domains (Fersht and Kaethner 1976; Perona and Hadd 2012), mutations at the amino acid binding domain are expected to decrease the binding affinity to amino acid without affecting tRNA binding. Such an aaRS mutant will synthesize aminoacyl-tRNA in a cognate amino acid concentration-dependent manner. Consequently, the aminoacyl-tRNA level required for normal cell growth can only be reached in the presence of elevated intracellular amino acid concentration, which is a label for amino acid overproducing cells. Based on this, we propose a new strategy for developing whole-cell amino acid biosensors. The original chromosomal copy of an aaRS is replaced by a mutant with decreased binding affinity to its cognate amino acid. The availability of the intracellular cognate amino acid shows positive relation to cell growth. Low-level amino acid producing cells show growth defect due to the insufficient aminoacyl-tRNA pool, whereas high-level amino acid producing cells show growth advantage due to the sufficient aminoacyl-tRNA pool (Scheme 1).

3.2. Proof-of-concept: isoleucine specifically relieved the growth defect caused by low-affinity isoleucine-binding IleRS mutant

A whole-cell isoleucine biosensor was developed as a proof-of-concept. The IleRS^{G94R} mutant with an elevated K_m value to isoleucine



Scheme 1. Schematic diagram of developing a whole-cell biosensor based on an aminoacyl-tRNA synthetase (aaRS) mutant. (A) Synthesis of the aminoacyl-tRNA and protein in normal cells. (B) The low-affinity aaRS mutant cannot produce enough corresponding aminoacyl-tRNA, which retards the protein synthesis and cell growth. (C) High amino acid supply restores the synthesis of aminoacyl-tRNA and growth of the cells with the aaRS mutant.

(33 mM) (Clarke et al., 1988) was used to replace the original IleRS with a K_m of 3 μM in *E. coli* MG1655, generating strain MG1655M. As expected, MG1655M showed decreased growth in TM medium compared with the wild-type strain MG1655. Twenty proteinogenic amino acids were individually added to TM medium to a final concentration of 0.5 g/L to test their effects on growth of MG1655M. The growth defect of MG1655M was specifically relieved by isoleucine addition but not other nineteen amino acids (Fig. 1). The results verified our concept that isoleucine can specifically restore the growth defect caused by equipment of an IleRS mutant with decreased binding affinity to isoleucine, which hints a possible strategy using IleRS based whole-cell biosensor to couple the isoleucine production capability with the cell growth.

3.3. Examination and extension of the isoleucine-dose-responsive range of the whole-cell biosensor

The growth response of MG1655M to different isoleucine doses (0–3.5 g/L) was then examined. With isoleucine addition increased from 0 to 2 g/L, growth of MG1655M gradually increased. The highest fold change in cell density reached 2.01-fold when 2 g/L isoleucine was added, compared with that without isoleucine addition. Further increasing the isoleucine concentration did not improve cell growth but slightly retarded the growth (Fig. 2). Such inhibition caused by a superfluous amino acid supply has also been noticed in a previous study (Avcilar-Kucukgoze et al., 2016). As for the wild-type MG1655 strain, the effects of supplementary isoleucine on cell growth was not as significant as those for MG1655M (Fig. 2). The results indicate that the growth of MG1655M is responsive to extracellular isoleucine doses below 2 g/L. However, a broad isoleucine-dose responsive range is preferred for the practical application of whole-cell biosensors.

To improve the responsive range, an idea to lower the intracellular isoleucine concentration by enhancing isoleucine export was tested. We overexpressed a branched-chain amino acid exporter YgaZH that can export isoleucine (Park et al., 2012), using three constitutive promoters P_{J23116}, P_{J23106}, and P_{J23100} with gradually increased strength. The resultant strains were named as MG1655MY1, MG1655MY2 and MG1655MY3, respectively. Strain MG1655MYC harboring an empty plasmid was also constructed and used as a control. High concentrations

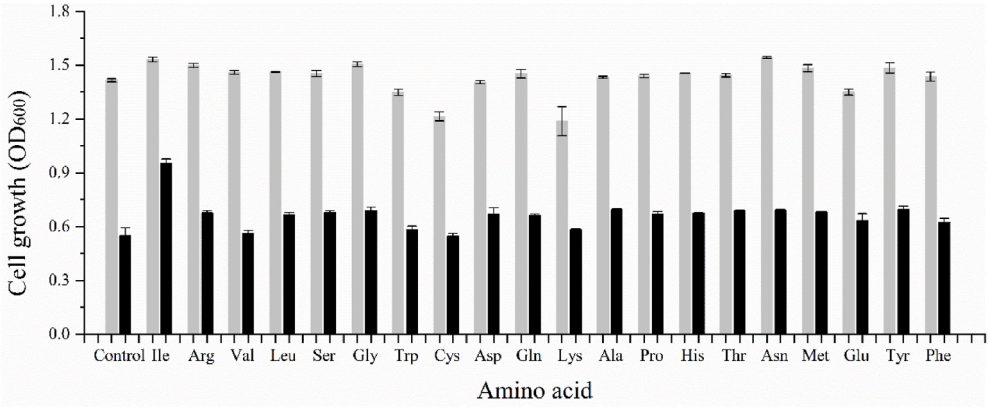


Fig. 1. Effect of proteinogenic amino acid on cell growth of strains MG1655 and MG1655M in TM medium. Each amino acid was supplied at 0.5 g/L, and cell growth without additional amino acid served as a control. The OD_{600} was measured at 4 h. Grey bars represent growth of MG1655, and black bars represent growth of MG1655M. Data are shown as the mean and standard deviation of independent triplicates.

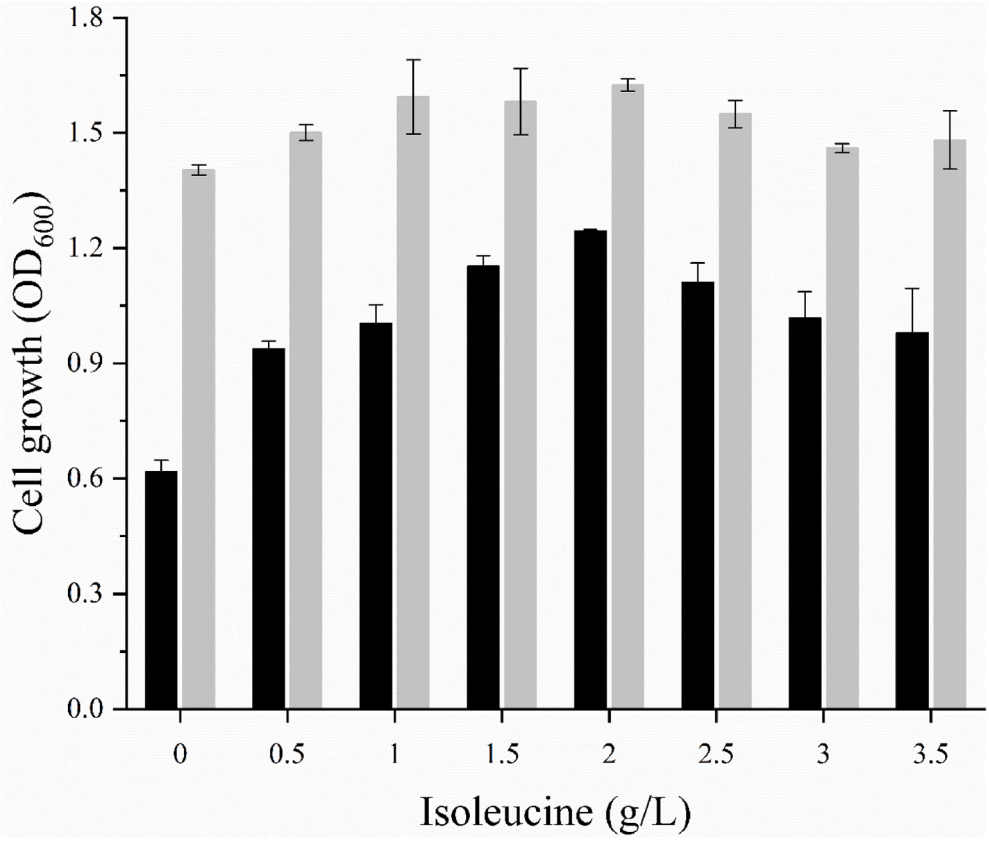


Fig. 2. Growth of MG1655M and MG1655 in TM medium with different concentrations of isoleucine. Black bars represent growth of MG1655M, and grey bars represent growth of MG1655. Data are shown as the mean and standard deviation of independent triplicates.

of isoleucine up to 16 g/L was added in TM medium to test the effect of YgaZH overexpression on intracellular isoleucine concentration and cell growth. All tested strains accumulated higher concentrations of isoleucine *in vivo* with increased isoleucine addition. However, the intracellular isoleucine of strains overexpressing YgaZH remained at lower levels in comparison to the control strain. With 2 g/L isoleucine addition, the intracellular isoleucine concentrations of MG1655MY1, MG1655MY2, and MG1655MY3 were 64%, 50% and 34% of that of MG1655MYC (1.31 ± 0.10 g/L), respectively. Strains with higher expression levels of YgaZH were found to have lower intracellular isoleucine concentrations (Fig. 3A), suggesting the successful YgaZH overexpression.

As for growth effect, similar trend was observed when cells were exposed to 0, 2, and 4 g/L isoleucine. Compared with the control strain MG1655MYC, strains with increased expression of YgaZH showed retarded cell growth. A negative correlation was observed between the expression level of YgaZH and cell growth rate. With 2 g/L isoleucine addition, the cell densities of MG1655MY1, MG1655MY2, and MG1655MY3 were 95%, 76%, and 59% of MG1655MYC (Fig. 3B and Fig. S1). For MG1655MYC, the cell density increased from 0.59 ± 0.02 without isoleucine supplement to a maximum of 1.31 ± 0.04 with 2 g/L isoleucine, which was a 2.22-fold improvement (Fig. 3B).

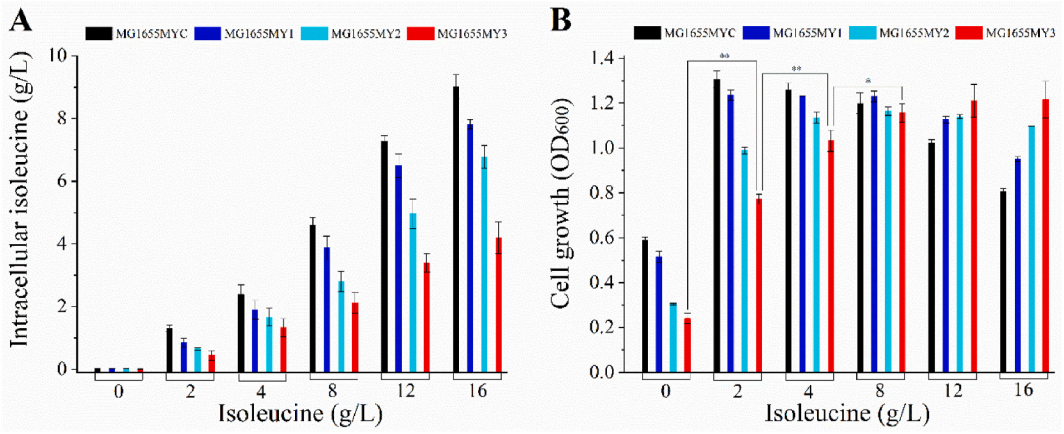


Fig. 3. Intracellular isoleucine concentration and growth of MG1655MYC, MG1655MY1, MG1655MY2, and MG1655MY3 in TM medium supplemented with different concentrations of isoleucine. (A) Intracellular isoleucine concentration after 4 h incubation. (B) Cell growth after 4 h incubation. Data are shown as the mean and standard deviation of independent triplicates. The *p* value was calculated by Student's two-tailed *t*-test (*, $p < 0.05$; **, $p < 0.01$).

Comparatively, the cell density of MG1655MY3 was improved by 3.21-fold with 2 g/L isoleucine addition and the fold change increased to 4.83-fold with 8 g/L isoleucine addition, indicating that the isoleucine response range was significantly increased by high-level YgaZH expression in MG1655MY3.

3.4. Endogenous isoleucine overproduction restored the growth of strains equipped with the isoleucine biosensor

We further examined whether endogenously produced isoleucine had the same growth recovery effect for strains equipped with the $IleRS^{G94R}$-based biosensor. Strain MG1655MY3 was used as the chassis for constructing isoleucine overproducers because of its satisfactory isoleucine response range. Strains Ile1 and Ile2 overexpressed the wild-type *ilvA* and the isoleucine-resistant *ilvA* mutant (C1339T, G1341T, C1351G, T1352C) (Park et al., 2012) via a plasmid, respectively. The chromosomal copy of *ilvEDA* operon was further overexpressed by replacing its native promoter to a strong promoter P_{trc} in strain Ile2, producing strain Ile3. A control strain Ile0 was constructed by transformation of an empty plasmid. The isoleucine production abilities of the four strains were in the order of Ile0<Ile1<Ile2<Ile3 (Fig. 4A). The intracellular isoleucine concentrations of the four strains were in the same order (Fig. 4B), suggesting higher-level isoleucine producers accumulated more isoleucine *in vivo*. The growth of these strains was then compared. The four strains showed growth rates in the order of Ile0<Ile1<Ile2<Ile3 in liquid TM medium, which was consistent with the order of isoleucine production capabilities (Fig. 4C). The results suggest that the isoleucine production level and the growth of strains equipped with the isoleucine biosensor were positively correlated. Thus, it is feasible to enrich isoleucine overproducers in liquid culture. Fig. 4D shows that the size of the colonies grown on TM agar plates can be obviously distinguished and correlated with isoleucine producing capabilities. The average colony areas of Ile1, Ile2, and Ile3 were 5.30-, 11.34-, and 14.48-fold larger than that of Ile0, respectively. Therefore, direct selection of desired overproducers on agar plates according to the colony size should also be feasible.

3.5. Selection of isoleucine producing strains from a mixture of producing and nonproducing strains

To test the efficiency of the whole-cell biosensor system, isolation of isoleucine producing strains from a mixture containing producing and nonproducing strains was performed by using the established colony-size-based method. Strain Ile1 was selected to mimic a target overproducer, whereas Ile0 was regarded as a nonproducer. Ile1 and Ile0 were mixed at a ratio of $1:10^4$ since desired candidates are always rare in a real screening scenario. The mixture was spread over TM agar plates as a selection and nutrient-rich LBI agar plates as a control. After 30 h-cultivation, the LBI plates were completely covered by bacterial lawns and no colony can be distinguished (Fig. 5A). However, a few colonies clearly outgrew others to a larger size on the TM agar plate (Fig. 5B). Ten large colonies on the TM agar plate were randomly picked, and all of them were identified as Ile1 strains by sequencing the plasmid. The results demonstrate the potential of the whole-cell biosensor system in high-throughput selection of isoleucine overproducers.

3.6. High-throughput selection of isoleucine overproducers from random mutation libraries

Next, we tested the usability of the whole-cell biosensor in high-

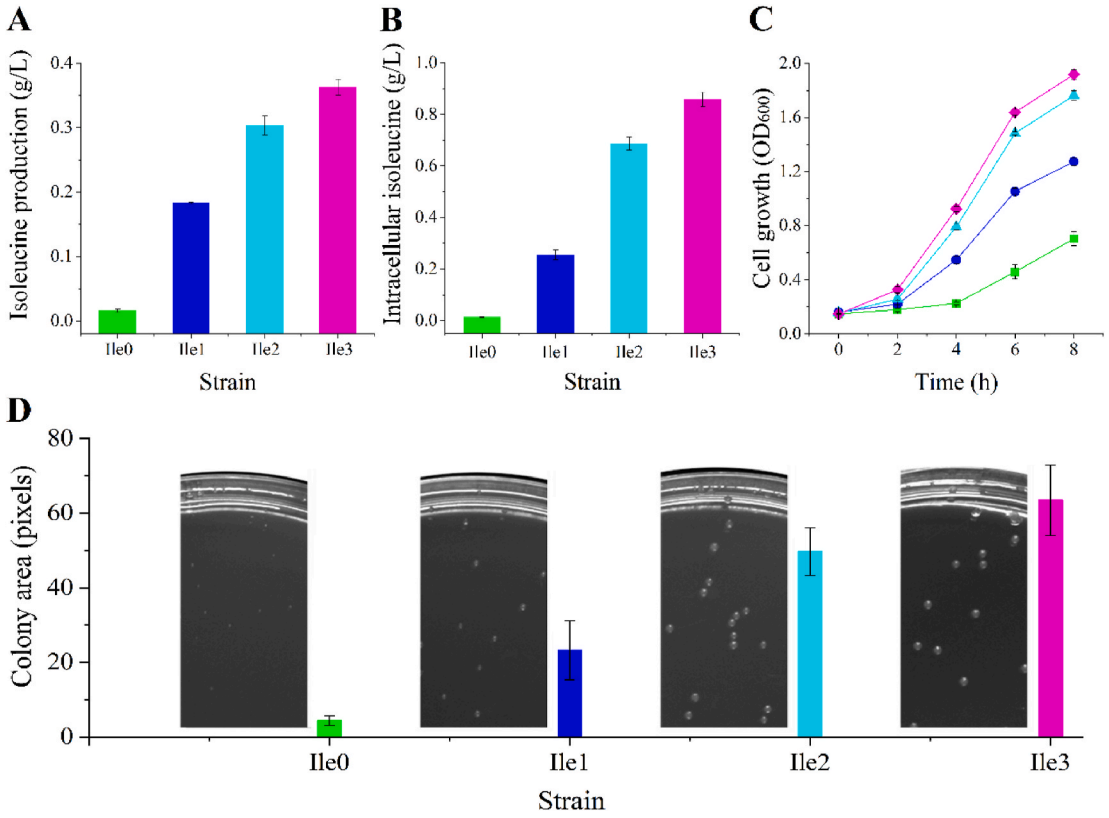


Fig. 4. Isoleucine production and growth of *E. coli* strains Ile0, Ile1, Ile2, and Ile3 equipped with the isoleucine biosensor. Lines or bars in green, blue, cyan, and magenta represent strains Ile0, Ile1, Ile2, and Ile3, respectively. (A) Isoleucine production in liquid TM medium. (B) Intracellular isoleucine concentration at 4 h. (C) Cell growth in liquid TM medium. (D) Cell growth on TM agar plates. The colony areas were calculated as pixels and the mean and standard deviation of ~200 colonies on independent triplicate TM agar plates are shown. Other data are shown as the mean and standard deviation of independent triplicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

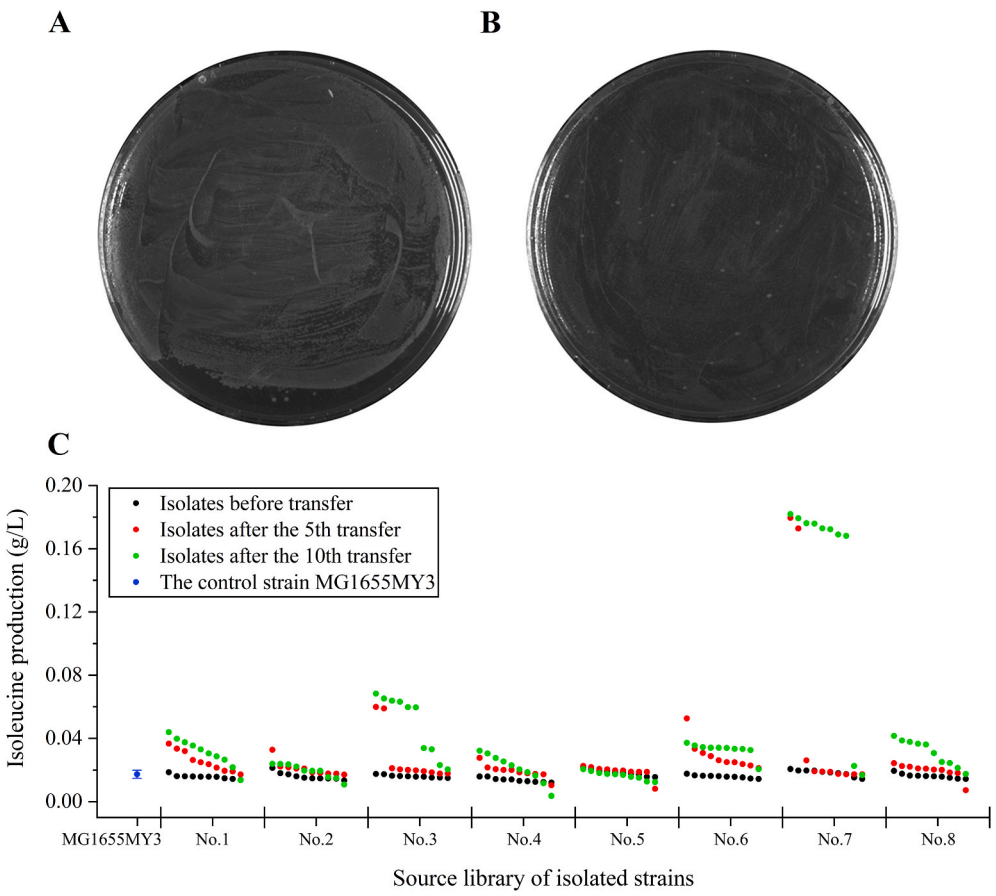


Fig. 5. Screening of isoleucine overproducers. (A) Growth of Ile0 and Ile1 mixture on LBI agar plate. (B) Growth of Ile0 and Ile1 mixture on TM agar plate. Ile1 and Ile0 were mixed at a ratio of $1{:}10^4$. The plates were incubated for 30 h until clear colonies appeared on the TM agar plates. (C) Isoleucine production of randomly isolated colonies from eight random mutation libraries during the iterative transfer process. Samples were taken from each library at the time points before transfer, after the 5th transfer, and after the 10th transfer and spread on LBI agar plates to obtain colonies. Ten colonies were randomly selected and subjected to isoleucine production assay. Strain MG1655MY3 was used as a control. The isoleucine titer of MG1655MY3 is shown as the mean and standard deviation of independent triplicates.

throughput selection of isoleucine overproducers from random mutation libraries. Desired mutants with a high isoleucine production level usually account for an extremely small portion of random mutation libraries. In this case, an enrichment of isoleucine overproducers in liquid medium is preferred. To generate random mutation libraries, eight parallel cultures of MG1655M were treated with UV light to introduce random mutagenesis. About 10^5 living cells from each culture were transformed with plasmid pTrcD-P_{J23100}-*ygaZH* to avoid unwanted mutations to the *ygaZH* cassette. The eight libraries were subjected to ten iterative transfers, each with 8 h-cultivation for enriching potential fast-growing isoleucine overproducers. Samples were taken from eight libraries after the 5th and 10th transfers, and spread on LBI agar plates. Then, ten colonies from each sample were randomly selected and cultivated for isoleucine production test. Before the iterative transfer enrichment, all tested colonies showed similar isoleucine production to that of the control strain MG1655MY3 (Fig. 5C). Colonies with improved isoleucine production began to appear after the 5th transfer. After the 10th transfer, five out of eight libraries (No.1, No.3, No.6, No.7, and No.8) contained isolates producing more than 2-fold isoleucine than the control strain MG1655MY3. Eight best isolates from No.7 library produced up to 0.17 g/L isoleucine, with a dramatic 10.75-fold improvement relative to MG1655MY3 (Fig. 5C). The results demonstrate the effectiveness of the whole-cell biosensor system for high-throughput selection of isoleucine overproducers from random mutation libraries.

4. Discussion

Biosensor-assisted amino acid monitoring is playing increasingly important roles in high-throughput selection of amino acid overproducers. Although traditional amino acid biosensors based on transcription factors, riboswitches, and fluorescence resonance energy transfer systems have been successfully developed (Binder et al., 2012; Choi et al., 2013; Liu et al., 2015; Mohsin et al., 2013; Mohsin and Ahmad 2014; Niazi et al., 2008; Wang et al., 2016; Yang et al., 2013), problems such as the poor ligand specificity (Lange et al., 2012; Lee and Wendisch 2017; Wang et al., 2017) and the unavailability of biosensors for many amino acids still exist. Amino acids are special metabolites that are directly involved in protein translation, a vital process for organism. Recently, amino acid biosensors based on the translation of reporter proteins were developed. A cell-free biosensor used amino acid deficient cell-free protein synthesis systems to generate fluorescence signals in response to exogenous amino acids, enabling rapid quantification of amino acids *in vitro* (Jang et al., 2017). Another whole-cell biosensor used installation of synonymous rare codons in the coding sequence of fluorescent proteins or antibiotic-resistant markers to hinder their translation. Therefore, amino acid overproducers could be identified because a sufficient supply of corresponding amino acid accelerated the translation of reporter protein. However, the rare tRNAs are easily charged in amino acid overproducers, which might limit the responsive dose to a relatively narrow range (Zheng et al., 2018).

In this study, we proved the concept of using mutant aaRSs with decreased affinity to its cognate amino acid to develop a new kind of whole-cell amino acid biosensors with universality and specificity. The $K_{m\ (Ile)}$ of $IleRS^{G94R}$ mutant used in this study is ~11000-fold higher than the wild-type IleRS (33 mM vs. 3 μM), whereas its affinities for ATP and $tRNA^{Ile}$ and apparent V_{max} are not significantly altered (Clarke et al., 1988). Previous studies have reported lysine (Commans et al., 1995), methionine (Ghosh et al., 1991), valine (Anderson and Neidhardt 1972), glycine (Folk and Berg 1970), and alanine (Theall et al., 1977) aaRSs mutants with increased K_m values, providing available aaRS devices for building various whole-cell biosensors. An effective and convenient method for obtaining new aaRS mutants is screening amino acid

auxotrophy caused by aaRS mutations (Gorini and Kaufman 1960; Iaccarino and Berg 1971). To specifically disturb the binding of aaRS with its cognate amino acid without affecting other functions, mutagenesis can be targeted to the amino acid binding domain, such as the N-terminal Rossman domain of the eleven Class I aaRSs including IleRS (Perona and Hadd 2012). The ligand specificity of aaRSs is the main principle for the fidelity of protein synthesis and is essential to cell survival (Perona and Hadd 2012), making specificity an intrinsic superiority of this kind of biosensors. Our results also show that the developed isoleucine biosensor specifically responds to isoleucine but not other nineteen amino acids.

The dose-response dynamic range is also essential for the practicability of a biosensor in high-throughput selection (Mannan et al., 2017). Engineering of the expression of the sensing part or signal gene is a usually used strategy. Even with optimization, the dose ranges of amino acid biosensors are mostly between 0 and 30 mM, and the signal output ranges are between 1 and 4 fold (Binder et al., 2012; Li et al., 2019; van Sint Fiet et al., 2006; Yang et al. 2013, 2017; Zheng et al., 2018). In this study, we proved that enhancing amino acid excretion by overexpressing the exporter is also a useful strategy. The maximum extracellular isoleucine dose was increased up to ~61 mM (8 g/L), with a 5-fold change in growth output. Since amino acid exporters widely exist (Hirasawa and Shimizu 2016), enhancing excretion should also be useful for engineering other amino acid biosensors.

Different from previously reported biosensors, the aaRS-based whole-cell biosensor can directly convert the amino acid concentration information into cell growth signal without the need of a signal gene such as the fluorescence gene or antibiotic resistance gene, which could avoid curing of the signal genes from the screened cells. Moreover, even the chromosomal aaRS mutant could be kept after selection because it could cause a synthetic addiction effect through arresting growth of nonproducing cells (Rugbjerg et al., 2018; Yang et al., 2019). Our results show that maintenance of the $IleRS^{G94R}$ mutant allowed stable isoleucine production during a sequential cultivation (Figs. S2 and S3).

False-positives cannot be completely avoided in application of biosensors, but can be relieved by using assistant methods (Raman et al., 2014). In this study, expected isoleucine overproducers were not enriched for some random mutation libraries during serial cultivation. To analyze the reasons, five colonies were randomly isolated from each library after the 10th transfer, and the $ileRS^{G94R}$ gene and the plasmid-borne *ygaZH* cassette were sequenced. Three colonies from No.2 library have the same mutation in the P_{J23100} promoter of *ygaZH*, and four colonies from No.5 library have another mutation in the P_{J23100} promoter (Fig. S4). These mutations might affect *ygaZH* expression and decrease isoleucine excretion, leading to the enrichment of false-positive cells. To avoid mutations in the *ygaZH* cassette and eliminate the false-positives, an assistant process, such as overexpressing the exporter gene using an easily cured temperature-sensitive plasmid and regularly replacing it with a fresh plasmid, is suggested.

5. Conclusions

In this study, a whole-cell biosensor specifically responding to isoleucine was constructed by using the $IleRS^{G94R}$ mutant with a decreased binding affinity to isoleucine. Isoleucine exporter YgaZH was overexpressed to tune the dose-response dynamic range. The developed biosensor was applied to screen isoleucine overproducers from random mutation libraries. The production levels of screened isoleucine overproducers are comparable to those of leucine, arginine, and serine overproducers screened by using other biosensor-based strategies (Mahr et al., 2016; Mustafi et al., 2012; Zheng et al., 2018). Although the isoleucine titers obtained here are still much lower than that produced by systematically engineered strains (Yin et al., 2014), an omics analysis of the screened mutants might provide new clues for isoleucine overproduction mechanism and development of hyperproducing isoleucine strains. Considering the twenty proteinogenic amino acids and many non-canonical ones have their specific aaRSs (Tian et al., 2020), the strategy presented in this study opens a new way to construct whole-cell amino acid biosensors.

CRediT authorship contribution statement

Xue Sun: Conceptualization, Investigation, Methodology, Formal analysis, Data curation, Visualization, Writing - review & editing. **Qinggang Li:** Conceptualization, Investigation, Methodology, Formal analysis, Data curation, Visualization, Writing - review & editing, Funding acquisition. **Yu Wang:** Conceptualization, Methodology, Formal analysis, Data curation, Writing - review & editing. **Wenjuan Zhou:** Conceptualization, Data curation. **Yanmei Guo:** Resources, Data curation. **Jiuzhou Chen:** Writing - review & editing, Formal analysis. **Ping Zheng:** Conceptualization, Methodology, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Jibin Sun:** Conceptualization, Methodology, Supervision, Project administration, Funding acquisition. **Yanhe Ma:** Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix B. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.bios.2020.112783.

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