Part II Biosynthesis of γ-PGA with tailored L/D glutamate ratio

Materials and methods

2.1 Strains and plasmids

The plasmid pZM1 and pDXW were used for gene cloning and plasmid construction (Zha et al., 2018).

2.2. Construction of plasmids and recombinant strains

The genes of *eGFP* and *mRFP1* were synthesized by biological company. The genes were then cloned into plasmid pZM1 and assembled in a monocistronic form using a previously published method (Xu et al., 2012). The transformation of plasmids into *C. glutamicum* was carried out following a previously published protocol (van der Rest et al., 1999).

2.3. Construction of racE plasmid series

The modified pZM1-*racE* plasmids containing two copies of lacO were constructed using a protocol previously established (Xu et al., 2012). The fragment covering lacO, a ribosome-binding site (RBS), and *racE* was amplified by PCR, digested by NheI/KpnI and cloned into XbaI/KpnI digested pZM1, resulting in the pZM1-*racE* plasmid with two copies of lacO. The transformation of plasmids into *C. glutamicum* was carried out following a previously published protocol.

Table 3 Primers used in Part II.

Primers	Sequence (5' to 3')	Notes
Bs-RacE-F-NdeI	TATACATATGTTGGAACAACCAATAGGAGTC	Clone the gene *BsracE* and
Bs-RacE-R-Bam	CGCGGATCCCTATCTTCTAATCGGTTCTTGCAGTG	construction of the
HI		recombinant plasmids
16S rRNA-F	ATATCAGGAGGAACACCAAT	Be used as the reference
16S rRNA-R	ACTACCAGGGTATCTAATCC	gene to normalize the data

RacE rRNA -F	ATCGCATTGGAAGACATC	Transcriptional-Level
RacE rRNA-R	TGCTCTTAATCGTATTCTCTG	Assay of *racE* from *B. subtilis*

2.4 Construction of eGFP and mRFP1 plasmid series

The fragments of *eGFP* and *mRFP1* were amplified by PCR, and cloned into digested pDXW, resulting in the pDXW-*eGFP* and pDXW-*mRFP1*. Different sequences of RBS were predicted by RBS Calculator and then synthesized by biological company. Finally, different sequences of RBS were cloned into digested pDXW-*eGFP* and pDXW-*mRFP1*, resulting in the pDXW-RBS-*eGFP* and pDXW-RBS-*mRFP1*. The fragment covering bicistronic structure was amplified by PCR from pDXW-BCD which was stored in the laboratory, and cloned into pDXW-*eGFP* and pDXW-*mRFP1*, resulting in the pDXW-BCD-*eGFP* and pDXW-BCD-*mRFP1*.

2.5 Construction of BCD-*racE plasmid series*

The fragment covering bicistronic structure was amplified by PCR, digested by XbaI/NdeI and cloned into XbaI/NdeI digested pZM1-*racE*, resulting in the pZM1-BCD-*racE*. Next, the fragment of BCD-*racE* was amplified by PCR, digested by AvrII/SalI. PCR products were inserted into the *Nhe*I and *Sal*I site of pZM1-*capB*CA*, resulting in the pZM1-*capB*CA*-BCD-*racE*.

2.6 Medium and cultivation methods

The medium and cultivation methods of *C. glutamicum* and its recombinant strains are described above.

2.7 Analytical procedures

2.7.1 Fluorescence intensity characterization

During the detection process, the cells were washed using PBS and the cells were diluted by appropriate multiples according to the measurement range of the instrument. The growth state and fluorescence intensity of the cells were detected using 96-well plates. The red fluorescent protein excitation wavelength is 560 nm,

and the emission wavelength is 630 nm. We used the infinite 200Pro instrument to measure green and red fluorescent protein and OD_{600} measurement.

2.7.2 Transcriptional-level assay

Gene transcriptional levels were measured by real-time PCR. Specifically, the total RNA at log phase of γ-PGA fermentation (8, 24 h) was collected by liquid nitrogen grinding and extracted with TRIzol® reagent; cDNA was amplified by the RevertAid First Strand cDNA Synthesis Kit (Takala, China). The primers used for amplifying the corresponding genes are listed in Table 3, and 16S rRNA from *C. glutamicum* ATCC13032 was used as the reference gene to normalize the data. The gene transcriptional levels of *racE* were compared with the control strain (FC0) after normalization to the reference gene 16S rRNA. The reaction system of qPCR is 17 μL, including 1.25 μL cDNA, 8.5 μL SYBR Green Mix, 0.425 μL forward primer and reverse primer, as well as 6.4 μL RNase free steaming water. The reaction conditions are as follows: 50 °C, 2 min; 95 °C, 10 min, 1 cycle; 95 °C, 15 s; 60 °C, 1 min, 40 cycles. All the experiments were performed in triplicate.

2.7.3 Determination of the ratio of L- and D-glutamic acid in γ-PGA

The method used was adapted from a literature procedure (Cromwick and Gross, 1995). γ-PGA samples were dissolved in 2 M HCl and hydrolyzed at 121°C for 50 min. Thereafter, after cooling the reaction to room temperature, 2 M NaOH was added dropwise with stirring until the solution reached pH 7. Next, 100 μL of 1-fluoro-2-4-dinitrophenyl-5-L-alanine amide (FDAA, Marfey's Reagent) and 20 μL of a $NaHCO_3$ solution were added to 50 μL of a 15 mg/mL glutamic acid (L-/D-mixture) solution from hydrolysis of γ-PGA. The mixed solutions were incubated at 40°C for 1 h. Samples were then cooled to room temperature and 10 μL of 20 2M HCl was added and mixed thoroughly. The samples were diluted with 1 mL acetonitrile for HPLC analysis.

For HPLC analysis, 5/95(v/v) acetonitrile/ammonium acetate buffer (50 mM, pH 5.3) was used as the eluent at a flow rate of 0.6 mL/min. The separation was performed on a XBridge TM column C8 (Waters, 3.5 μm, 2.1×50 mm) at 40°C.

Detection of eluents was with a diode array detector at 340 nm.

Results

2.8 Modification of γ-PGA composition by heterologous expression of racE

In γ-PGA produced by the engineered *C. glutamicum* F343, the major monomer unit was L-glutamate as demonstrated by the observation that 97.1% of the glutamate units were L-glutamate(see Fig.4A). This is possibly due to the low intrinsic activity of glutamate racemase, which can convert L-glutamic acid to D-glutamic acid. To change the ratio of L-glutamate units in the product γ-PGA and investigate the effect of the L-glutamate supply with different ratios of L-glutamate and D-glutamate on γ-PGA production, the glutamate racemase gene *racE* derived from *B. subtilis* Ia1a was expressed with different transcriptional levels in the engineered strain *C. glutamicum* F343 pZM1-Ptac-*capB*CA* (strain FC0).

Real-time quantitative PCR was employed to measure the expression levels of *BsracE*. The transcriptional levels of *BsracE* in *C. glutamicum* F343 pZM1-*capB*CA*-(2lacO)*BsracE* (strain FC2), where two lacO operators were incorporated, were approximately 70.0% and 37.6% ($P<0.05$) of that in strain *C. glutamicum* F343 pZM1-*capB*CA*-(1lacO)*BsracE* (strain FC1) at 8 h and 24 h, respectively(see Fig.4A). Then the effect of different expression of *BsracE* on the ratio of L-glutamate in γ-PGA was monitored. As expected, 97.1% of the glutamate units is L-glutamate in the engineered strain FC0, and the percentage of L-glutamic acid in PGA decreased to 36.9% in the engineered strain FC1, while in engineered strain FC2, the ratio of L-glutamate monomer was decreased to almost half the PGA moieties (62.2%)(see Fig.4B). Thus, a large and even range of L-glutamate (36.9-97.1%) was achieved in the γ-PGA-producing *C. glutamicum* strains.

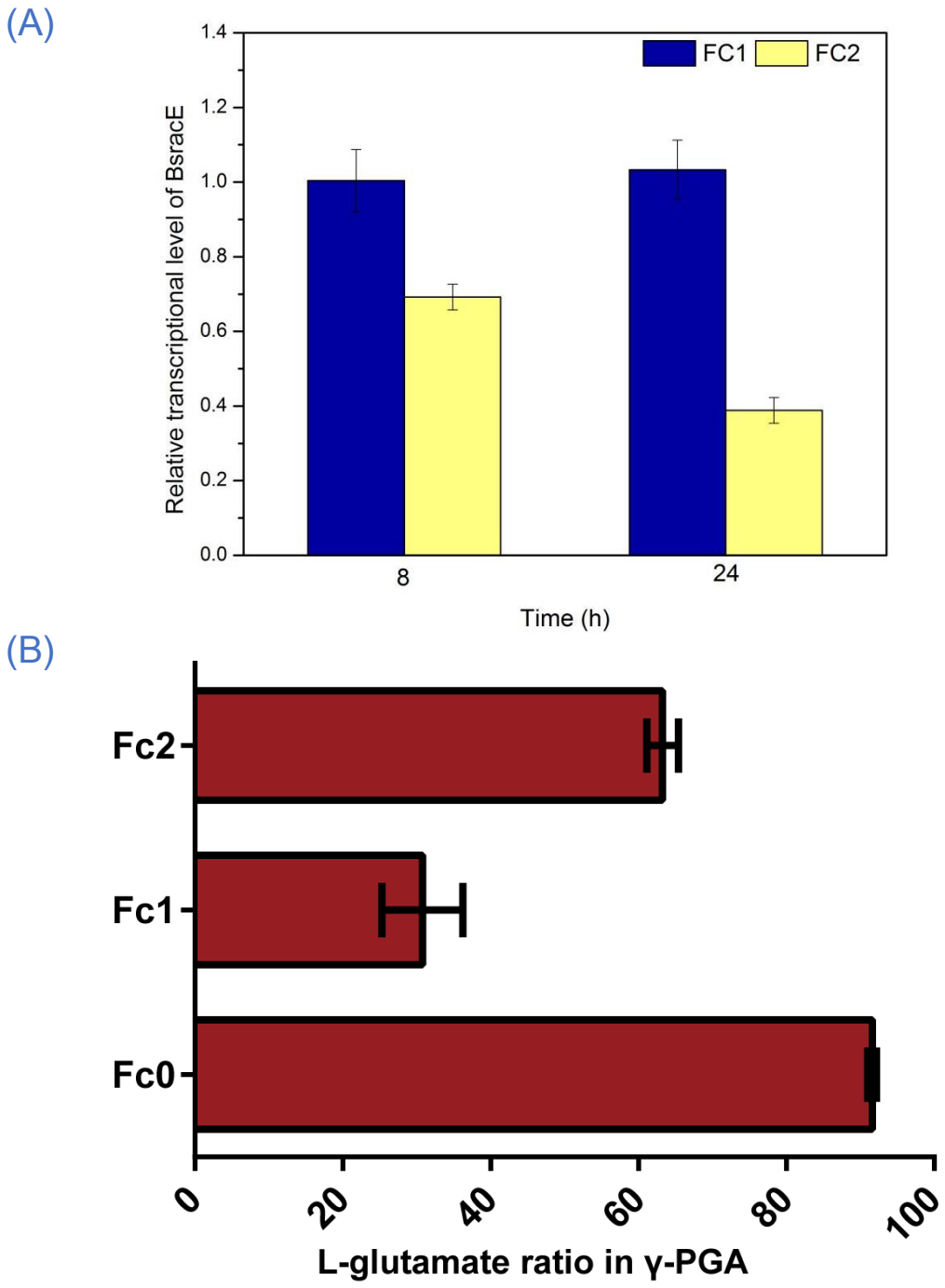

Fig.4. Modification of γ-PGA composition by regulating expression of heterologous *BsracE*

(A) Relative transcriptional level of *racE* in FC1 and FC2.

(B) L-glutamate ratio of γ-PGA produced by FC0, FC1 and FC2. FC0, *C. glutamicum* F343 pZM1-Ptac-*capB*CA*; FC1, *C. glutamicum* F343 pZM1-*capB*CA*-(1lacO)*BsracE*; FC2, *C. glutamicum* F343 pZM1-*capB*CA*-(2lacO)*BsracE*.

2.9 Fine-tune L-glutamate monomer ratio of γ-PGA

We selected six RBS from RBS Calculator. Sequence of expression intensity given by

RBS Calculator is: R18000 > R2000 > R200 > R180 > R20 > R18. According to the Figure.5, we had a conclusion that as the increasing of the predicted expression intensity of RBS given by RBS Calculator, the actual measured fluorescence intensity is not as general as predicted.

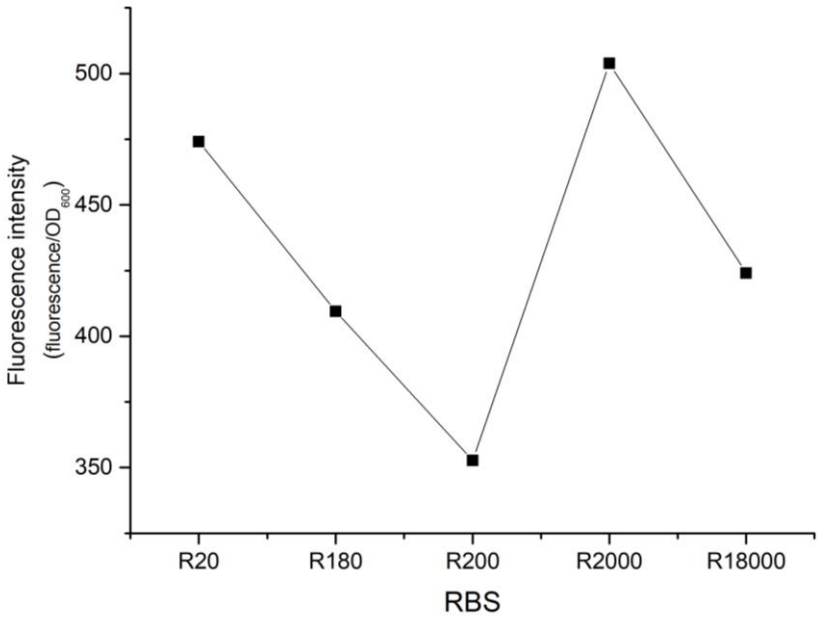

Fig.5. Fluorescence intensity measured by *Corynebacterium glutamicum*-pDXW-R20/180/200/2000/18000-*mRFP1*

This conclusion inspired us that it is difficult to regulate L-glutamate monomer ratio of γ-PGA precisely by changing RBS directly (Nishizawa et al., 2010). So Pro. Zhang instructed us that addition of the bicistronic structure can stable the expression intensity of RBS (Kimura et al., 2005; Nishizawa et al., 2010). Considering of the fact that the measurement of L-glutamate monomer ratio of γ-PGA is cumbersome and complicated, we came up with an idea that we can add different RBS with bicistronic structure in front of the *mRFP1*, and sequence the expression intensity of these RBS by measuring the fluorescence intensity.

After constructing *Corynebacterium glutamicum*-pDXW-BCD-R18/20/180/200/2000/18000-*mRFP1* and detecting fluorescence intensity of these strains, we reranked the expression intensity of six RBS. Sequence of expression intensity is: BCD-R180 > BCD-R18000 > BCD-R2000 > BCD-R18 > BCD-R20 > BCD-R200 (see Fig.5). After constructing *Corynebacterium glutamicum*-pDXW-BCD-R18/20/180/200/2000/

18000-*eGFP* and detecting fluorescence intensity of these strains, we found that after the addition of the bicistronic structure, the expression intensity of RBS is 1-50 times, and the same RBS with bicistronic structure have the same expression intensity of the two fluorescent protein genes.

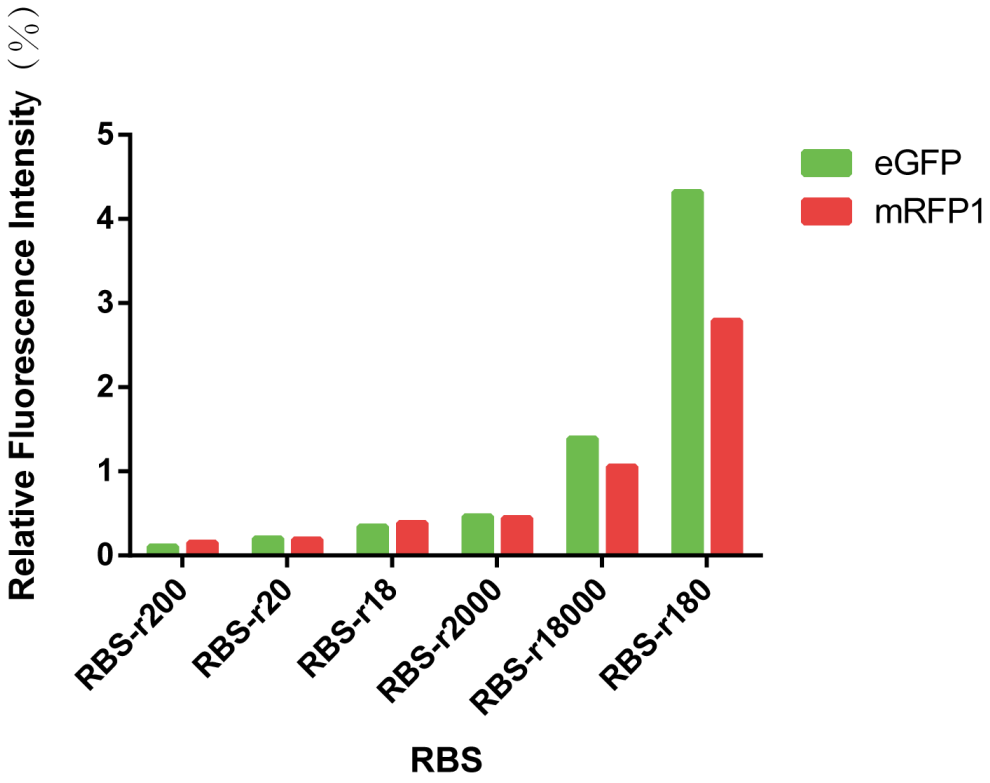

Fig.6. Relative value of fluorescence intensity measured by engineered *Corynebacterium glutamicum*

Green represents *Corynebacterium glutamicum*-pDXW-BCD-R18/20/180/200/2000 /18000-*eGFP*;

Red represents *Corynebacterium glutamicum*-pDXW-BCD-R18/20/180/200/2000 /18000-*mRFP1*.

After verifying that the bicistronic structure does have the function of stabilizing the expression intensity of RBS, we added different RBS with bicistronic structure in front of the racemase gene *BsracE* to obtain different racemase enzyme activities which can regulate L-glutamate monomer ratio of γ-PGA. At last, we can regulate L-glutamate monomer ratio of γ-PGA precisely. For the reason that measurement of

fluorescence intensity is easy and fast, we can first create a simple database by correspondence between several different RBS and corresponding L-glutamate monomer ratio of γ-PGA. Then we characterize the expression intensity of different RBS with bicistronic structure by reporter gene and infer its corresponding L-glutamate monomer ratio of γ-PGA by modeling to further improve the database.

So far, our team members still carry out relevant experiments. Unfortunately, due to time constraints, the results of these experiments have not been obtained. But we expect that in the future we or other igemers can continue our work and let the research go on.

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