

Pilot study on the chemical inducible promoter-biosensor to promote biosynthesis of valuable medicinal compound

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Feng Y, Wang B. Pilot study on the chemical inducible promoter-biosensor to promote biosynthesis of valuable medicinal compound. *J Exp Clin Microbio* 2019;2(2):1-13.

Medicinal compounds such as polyamine, sugars, antibiotic, lactams, amino acids, organic acids or redox molecules, play an important role in the human disease prevention and treatment. However, these compounds are not necessary to be chemically synthesized, which is complicated and hard, and sometimes can cause serious environmental pollution. Alternatively, microbial metabolic engineering is an ideal consideration for producing these molecules. In this paper, we preliminarily construct the chemical inducible promoter-biosensor with dynamic genetic regulatory circuit, and assemble the responsive promoters to promote the biosynthesis of putrescine with high efficiency in “push” and “pull” mode. The

Cgl1169 and Cgl1141 promoters of *Corynebacterium glutamicum* (*C. glutamicum*) that is indeed in responsive to L-Ornithine or putrescine has been screened by immunofluorescence detection (OD535/OD600) and presented good fitting responsive kinetic curve. This new approach can be applied to other promoter screening to construct a fantastic high-throughput platform to large scale screen chemical induced promoters, and to generate drug biosynthesis modular for its production. Thus, these newly fabricated biosensors that utilize the promoter with good properties will not only detect the medical toxic substances, but also can maximum biosynthesis efficiency of medicinal compound, which will further promote the development of polyamine synthetic microbiology.

Key Words: *Chemical inducible promoter-biosensor; High-throughput platform; Dynamic regulatory circuit; Medicinal compound; Toxic substances; Polyamine synthetic microbiology*

INTRODUCTION

Many valuable compounds are synthesized by chemical approach. Putrescine is molecular composition of polyamide of polymeric material (4, 6 PA46) that is chemically synthesized through the strict condensation reaction by dicarboxyl and putrescine [1]. However, the catalytic condition is very expensive and various reagents with strong toxicity and flammability are not environment-friendly, and human healthy threaten. So, microorganism offers another perspective to produce these diverse industrial and medicinal chemical, and materials from renewable resources. Although the microorganism can overcome the problems of chemical synthesis approach, they are not evolved to suit the high efficient production of given molecules, even is rather low. As a result, we should create the new pathways, new cellular regulatory circuit to develop various microbial cell factory or biosensors to efficiently manufacture the medicinal compound.

Putrescine is a well-known kind polyamine that exists in bioactive cells. The common biopolyamines are histamine, spermidine and spermine which are vital for in organizational metabolic activities, such as enhancement of DNA polymerase activity, promoting DNA synthesis and transcription, regulation of calcium channel activity *in vivo*, promotion of cell proliferation and growth, and participation of inflammation reaction of organism, repairing of lost tissue, etc. All these pharmacological activities can be used for clinical medical applications.

Because putrescine has so much medicinal value, how to engineer the putrescine biosynthesis pathway attracts wide attention for pharmacological researchers and biological scientists. Putrescine can be either directly conversion from L-Ornithine by decarboxylation in ODC biosynthesis pathway or conversion from L-Arginine by decarboxylation and deamination in ADC biosynthesis pathway. Animals only persist ODC biosynthesis pathway, however, many bacteria, plant and archaeobacteria persist both ODC and ADC biosynthesis pathway. The main difference of the biosynthesis pathway of putrescine between *E. coli* and *C. glutamicum* is the intermediate step: argE and argJ catalytic step, there is no feedback

regulation step of argJ, and intermediate catalytic argE step in *E. coli* (Figure 1).

It is reported that we could enhance expressing the *SpeC* gene to promote the conversion of L-Ornithine to putrescine with engineered gene promoter, in the mean time to block the ADC biosynthesis pathway and the de-composition pathway of n-acetyl-putrescine to increase putrescine production (Figure 1) [2-5]. The efflux protein PotE of putrescine can be enhanced to be expressed to promote the extracellular accumulation putrescine. So, with the development of the gene engineered technique and the basic biological science [6,7], many valuable medicinal compounds can be orderly and efficiently created by microbial metabolism engineered approaches.

MATERIALS AND METHODS

Bacterial culture medium

LB (1L): Tryptone 10 g, Yeast extract 5 g, NaCl 10 g, PH 7.0, 121 sterilization for 20 min; Solid medium added 15 g agar powder.

LBHIS (1L): Tryptone 5 g, Yeast extract 2.5 g, NaCl 15 g, Brain heart infusion (BHI) 18.5 g, D-Sorbitol 91 g, 116 sterilization for 20 min.

Drug and reagents

Chloramphenicol antibiotic (working stock 10 µg/mL), Restriction endonuclease was purchased from Thermo InC.

Gold Star T6 Super PCR Mix (1.1X) TSE101 were purchased from Beijing TsingKe Biotech Co. Ltd. Primer STAR

Max DNA polymerase, T4 DNA ligase and 5 KD DNA marker were purchased from TaKaRa InC. Taq DNA polymerase, dNTP, antibiotic were purchased from new probe InC, Guangzhou. Bacterial genome extraction Kit and DNA agarose gel recovery Kit were purchased from Magen InC.

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Received: 1 January 2019, Accepted: 25 February 2019, Published: 15 March 2019



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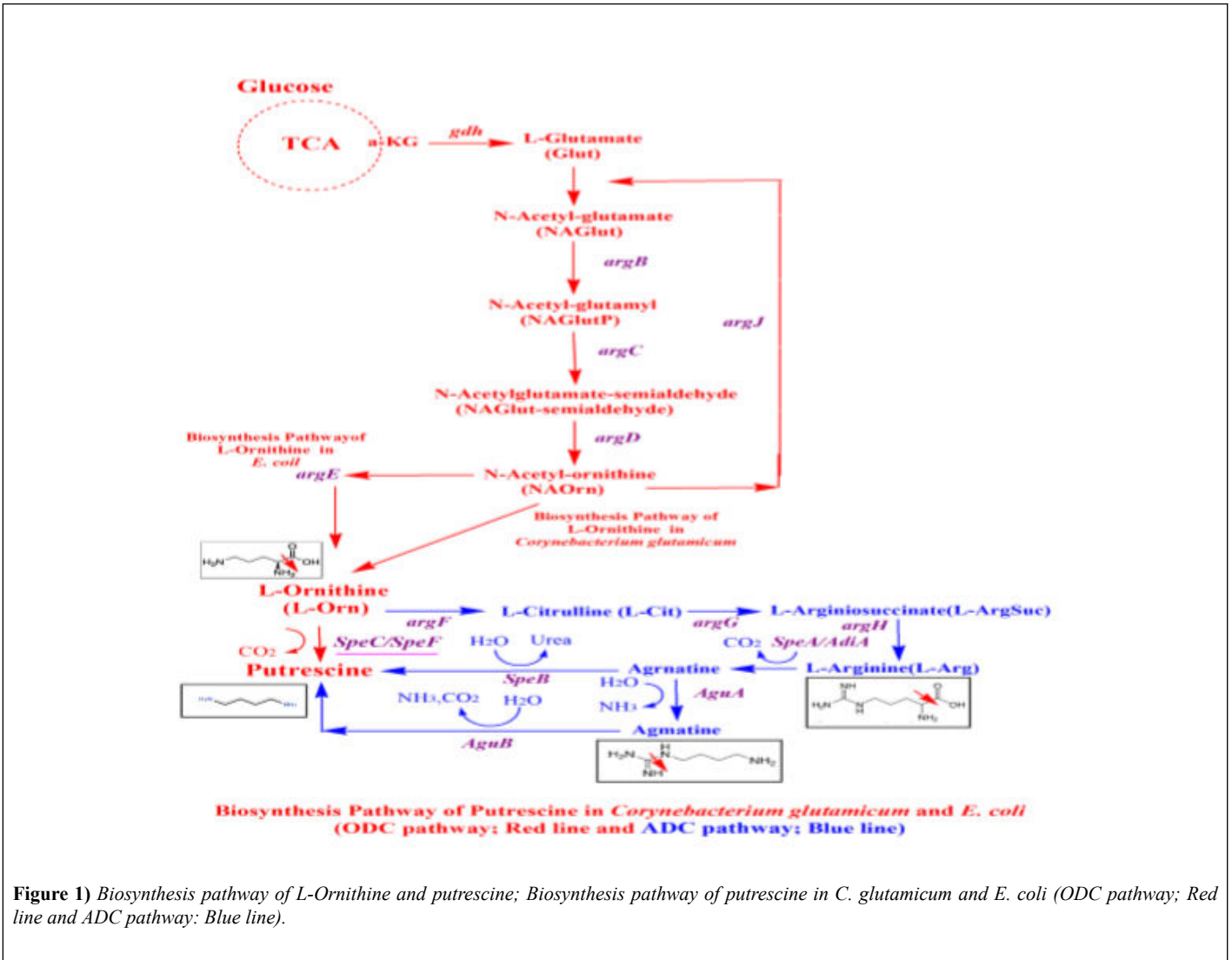


Figure 1) Biosynthesis pathway of L-Ornithine and putrescine; Biosynthesis pathway of putrescine in *C. glutamicum* and *E. coli* (ODC pathway; Red line and ADC pathway; Blue line).

Extracting the genome DNA of *C. glutamicum*: Transform 1.2 mL bacteria (*C. glutamicum*) culture medium to 1.5 mL centrifuge tube, 10,000 g/min centrifuge 1 min, harvest the cell culture. Add buffer 200 µL and lysozyme 30 µL to the cell precipitation, and vortex to suspend the bacteria. The following extracting procedures of the genome DNA of *C. glutamicum* according to the instructions of PureLink® Genomic DNA Mini Kit.

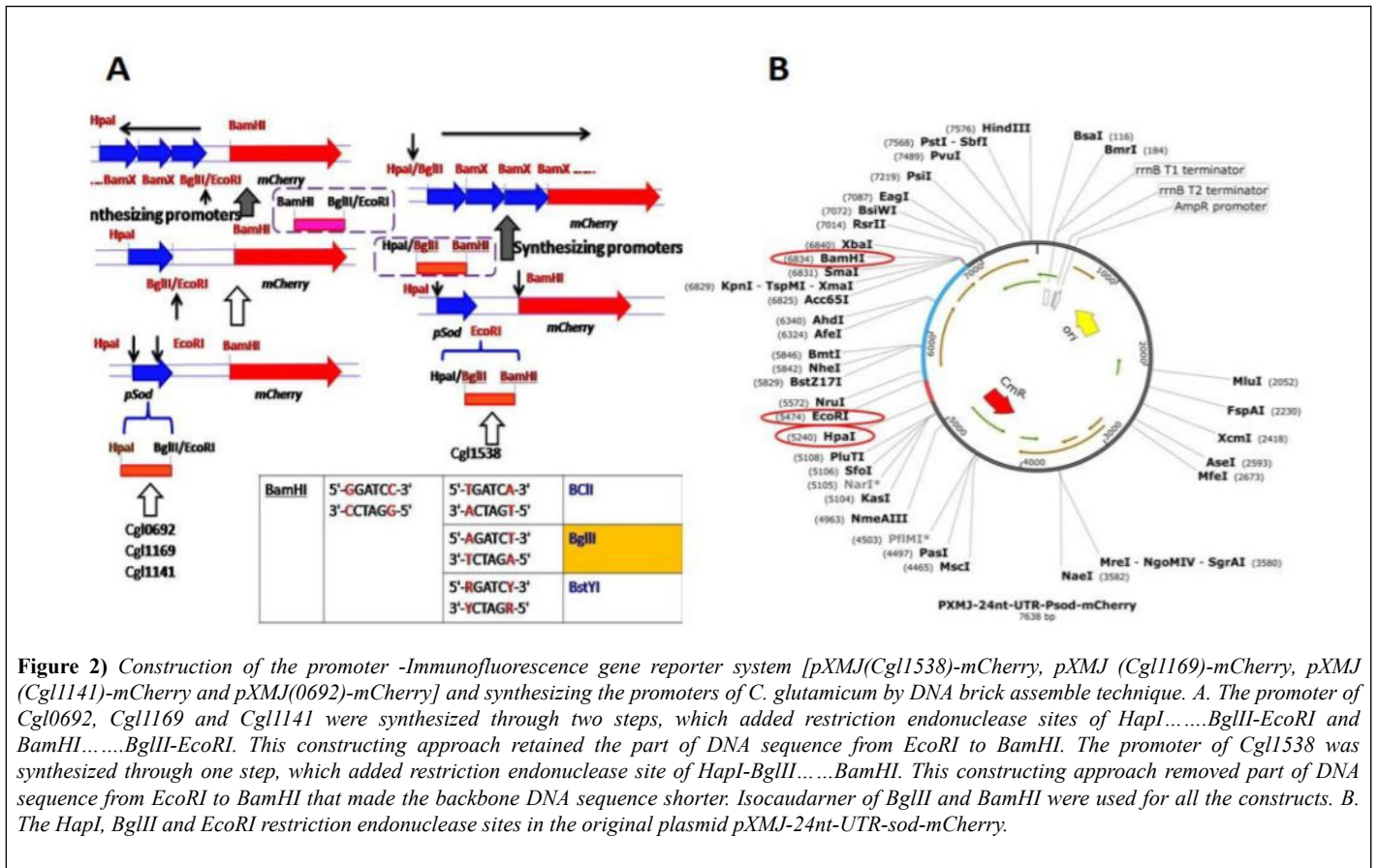
PCR the promoter sequence from the genome of *C. glutamicum*: We have designed the following primers to PCR Cgl1538, Cgl1169 and Cgl1141 and Cgl0692 promoters of *C. glutamicum*. The components of PCR reaction system was Gold Star T6 Super PCR Mix (1.1X) TSE101 45 µL, 10 µM forward primer 2 µL, 10 µM reverse primer 2 µL, 100 ng/µL genome DNA of *C. glutamicum* 1.5 µL, total volume is 50 µL. The PCR reaction condition was 98 2 min, 98 10 s, 56 10 s, 72 15 s (kb/10 s), 721 min, 35 cycles.

Construction of the promoter- Immunofluorescence gene reporter system: The restriction map of original plasmid pXMJ-24nt-UTR-sod-mCherry is represented in Figure 2. The restriction sites of HpaI (5240), EcoRI (5474) and BamHI (6834) presented in the map is the cloning sites of the promoters Cgl1538, Cgl1169, Cgl1141 and Cgl0692 (table 1) which are cloned fusing the immunofluorescence gene (mCherry gene). These plasmids are denoted as pXMJ(Cgl1538)-mCherry, pXMJ (Cgl1169)-mCherry, pXMJ (Cgl1141)-mCherry and pXMJ(0692)-mCherry, respectively. All the correct clones were confirmed by PCR and sequencing [8-12]. The synthesizing of these promoters can be further performed by brick assembled technique, which utilizes isocaudamer

ligation (BamHI/BglII) for connecting these promoters to enhance their property. The approach of synthesizing promoters of *C. glutamicum* is illustrated in Figure 2.

TABLE 1 Design the primers for PCR the promoters of Cgl1538, Cgl1169 and Cgl1141 and Cgl0692. Red and capitalized fonts in the primer stand for the restriction endonuclease sites; The non-capitalized font stands for the primer pairs for PCR the promoters of *C. glutamicum*.

Cgl1538(F-pATG)	F: 5' - ATTGTTAACAGATCTgtaaagtctaaagcttact-3'
Cgl1538(R-pATG)	R: 5' - CCGGGATCCcgtgacaccttttactgggt-3'
Cgl1169(F-pATG)	F: 5'- ATTGTTAACgcaaagcttattcggaaggc-3'
Cgl1169(R-pATG)	R: CCGGAATTCAAAAAGATCTcccgaaaatgctcgtagaa-3' 5'-
Cgl1141(F-pATG)	F: 5' - ATTGTTAACttcgatttctaatgctcata-3'
Cgl1141(R-pATG)	R: CCGGAATTCAAAAAGATCTgaatgctctctataaccattc-3' 5'-
Cgl0692(F-pATG)	F: 5'- ATTGTTAACgggggtgctcctctaaaaagcgaa - 3'
Cgl0692(R-pATG)	R: CCGGAATTCAAAAAGATCTgtgaaagccctctttggga-3' 5'-



Electrotransformation of pXMJ (Cgl1538, Cgl1169, Cgl1141, Cgl0692)-mCherry plasmids to *C. glutamicum* mutant(s) of ER6937R42, 16-17-CPVF-ALE and ATCC13032 wild type(WT) (*C. glutamicum* mutant ER6937R42: highly produce L-Ornithine; *C. glutamicum* mutant 16-17-CPVF-ALE: highly produce putrescine): 20 μ L sterile water dissolves the plasmids of pXMJ (Cgl1538)-mCherry, pXMJ (Cgl1169)-mCherry, pXMJ (Cgl1141)-mCherry and pXMJ (Cgl0692)-mCherry. 100 μ L competent cells of *C. glutamicum* mixed with 3 μ L plasmid DNA in the pre-cold Gene Pulser®/MicroPulser™ 0.1 cm electroporation cuvette were cooled on ice for 10 min. Immediately, 800 μ L LBHIS culture medium was added into the electroporation cuvette after 2.5 kv electric shock for 4 ms/each. Blow up and down for several times and transformed all the electroporated bacterial culture into 1.5 mL centrifuge tube. The bacterial culture was incubated in water at 466 min and statically cultured at 30 for 3 days. After that, the bacterial culture was centrifuged at speed of 5000 rpm/min for 2 min, the supernatant was discarded and then 100 μ L re-suspended bacterial precipitation was retained to uniformly spread the bacterial strain onto the LBHIS solid medium plate [13-15]. The bacterial culture was inverted culturing at 30 for 3 days.

Characterization of the activity of the promoter

The activity of the promoter can be characterized by selecting 3 random *C. glutamicum* clones to check the ratio OD535/OD600 (Detected at different time point of 0, 24, 48, 72 h) or with an initial optical density value of bacterial density of OD600 of 0.2 (Detected at different time point of 0, 2, 4, 18, 26, 36, 42, 72 h). The time-dependent kinetic curve was constructed in these two analysis approaches.

Detecting the intracellular and extracellular concentration of L-Ornithine

Detecting extracellular concentration of L-Ornithine: 1 mL *C. glutamicum* bacteria cultured was harvested from the selected random

clones or inoculating from the culture at the initial bacterial density OD600 of 0.2. All of the *C. glutamicum* was cultured for 3 days. After that, 1 mL cell culture was centrifuged at 12000 g/min for 20 min. 500 μ L supernatant was got to react with 500 μ L triketohydrindene hydrate, then incubated in boiling water for 1 h. Subsequently, the reaction solution was cooled to room temperature and 1.5 mL glacial acetic acid was added to terminate the reaction. 1.5 mL mixed reaction solution was detected under the light absorption value OD512.

Detecting intracellular concentration of L-Ornithine: The centrifuging precipitation was ultrasonicated after adding 500 μ L ddH₂O (Total time is 15 min, ultrasonicated 10 s, stop 5 s). The ultrasonicated solution was centrifuged at 12,000 g/min for 20 min, and 500 μ L supernatant was got to react with 500 μ L triketohydrindene hydrate. The subsequent procedure was the same as above.

Detecting the intracellular and extracellular concentration of putrescine

The putrescine concentration was determined using a Shimadzu HPLC system (LC-20A HPLC, Shimadzu, Japan) equipped with an Inertsil ODS-SP column (5 μ m, 4.6 mm \times 150 mm, GL Sciences InC., Tokyo, Japan) as described by Schneider and Wendisch [6-8]. Putrescine was first derivatized using 9-fluorenylmethyl chloroformate (FMOC). The fluorescent derivatives were detected by excitation at 263 nm (emission at 310 nm). The mobile phase consisted of solvent A (0.05 M sodium acetate, PH 4.2) and solvent B (acetonitrile) with a flow rate of 1.3 mL/min. The following gradient was used: 0 min, A-B (38:62, v/v); 5 min, A-B (38:62, v/v); 12 min, A-B (57:43, v/v); 14 min, A-B (57:43, v/v); 20 min, A-B (65:35, v/v); 25 min, 76% A-B (76:24, v/v); 35 min, A-B (76:24, v/v); 35.01 min, A-B (38:62, v/v); 45 min, A-B (38:62, v/v). A standard curve was constructed from serial dilution of a standard stock solution of putrescine.

RESULTS

Candidate promoters were selected based on the compared transcriptome of *C. glutamicum* genome

In the previous studies, the wild-type *C. glutamicum* biosensor strain (ATCC13032), variant strain over expressed L-Ornithine (ER6937R42)

and variant strain over expressed putrescine (16-17 CPVF-ALE) were cultured in LBHIS medium for 72 days. Subsequently, the total RNA of these strains were reverse transcribed to double strand DNA to check the transcriptome of these strains [9-12], which profiling the up-regulated genes. The genes that were highly expressed (LogFC ratio) and the candidate promoters were selected based on the transcriptome data (Table 2).

TABLE 2 The LogFC ratio of the compared transcriptome of *C. glutamicum* genome (LogFC: ER6937R42/ ATCC 13032WT > 6.0; 16-17 CPVF-ALE/ ATCC 13032WT > 5.0)

Gene ID	LogFC	Gene length	Gene symbol	Gene function
Gene 1150	7.83(ER6937R42/ ATCC13032WT) 7.28(16-17CPVFALE/ATCC 13032WT)	381	Cgl1169	Short chain fatty acids transporter, Invasion associated protein
Gene 681	6.79(ER6937R42/ ATCC13032WT)	1089	Cgl0692	G3E family GTPase
Gene 1519	6.46(ER6937R42/ ATCC13032WT) 5.67(16-17CPVFALE/ ATCC13032WT)	1815	Cgl1538	Cell wall-associated hydrolase
Gene 1124	5.46(16-17CPVFALE/ATCC 13032WT)	1413	Cgl1141	Molecular function: flavin adenine dinucleotide binding(GO: 0050660);molecular_function:N,N-di methylaniline monooxygenase activity

Search the genome library of *C. glutamicum* (NCBI) to find the promoter sequence of up-regulated genes. The promoter sequences of up-regulated genes (Table 2) were found in the genome library of *C. glutamicum* (NCBI). The promoter sequence and gene functions are listed in Table 3.

Experimental approaches require the coordinated control of multiple genes. While multiple systems have been developed for use in *C. glutamicum*. In order to study bacterial gene expression in *C. glutamicum*, we developed an original plasmid, pXMJ-24nt-UTR-sodmCherry, which confers constitutive *mCherry* gene expression from cloned promoters.

Corynebacterium glutamicum, a fast-growing and aerobic Gram-positive microorganism able to secrete large amounts of glutamate under suitable conditions, has a long history of use for the industrial production of various primary metabolites, including amino acids and nucleotides. The complete genome sequence of *C. glutamicum* ATCC 13032 has been published. Genome sequences have been reported for some other closely related microorganisms, such as *Corynebacterium efficiens* YS-31 and *Corynebacterium glutamicum*. Here we report the variant strains *Corynebacterium glutamicum* ER6937R42 and 16-17 CPVF-ALE), industrial producers generated by conventional mutagenesis which have been widely used in production of L-Ornithine and putrescine.

mCherry gene neither interferes with other plasmids harboring an intact expression system nor alters the growth of *C. glutamicum* during intracellular growth. Furthermore, the broad-host range plasmid backbone of *mCherry* gene allows constitutive gene expression in a wide variety of Gram-negative bacterial species, making *mCherry* gene a useful tool for the greater research community.

TABLE 3 The candidate promoter sequence of Cgl1538, Cgl1169, Cgl1141 and Cgl0692 was searched from the complete genome of *C. glutamicum* ATCC 13032 in NCBI

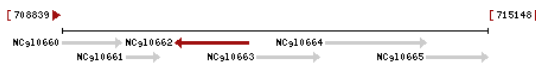
Promoter	Sequence	Location in the Chromosome (NCBI)	Gene function
Promoter: Cgl1538	<p>gtaaag ttctaaagct ttacttatag cacatttcggt aggtgtcagag aaaattccca aattgcggaattccacata ctttgcgcca caaagtgata aacatcaca aattcatga ctacgccattatgcaggcc agaacatgca aaacctgca cgctctggcc tttgttatg taactgttgaaagatgta tcaaaagtta ccaagggtgac ttaatgttca gatatcgact catgggttgtttccaaaac tgaatcaat tgagtaaca gtagttatca agcgttaaac ctgaaactccactcttt tactgatggg catttgggca aacggggaag cttgctggag atgcaataaatcggatgaatg gttgtgcaa tgaagaggag agtggcccat agttccgct acggaacgag gagtcttac gttcaattc ttgcaagta tctagcgtca aggctgata tgtgaaggattcggattga acaggagaac atgtggccaa tctagattca gttcggcctg ggggtcgggcagctcttca cgtaccata cccaggtaaa aggtgtcacg (586bp)</p>	<p>Cgl1538 promoter is the promoter of Ncgl1480 gene in the following genomic context.</p>	Cell wall-associated hydrolase(invasion-associated proteins)
Promoter: Cgl1169	<p>agcaaagct tattcgcgaa ggcatccaag gaacgtgggg attcattct caattcccgt tctacgcagg cattttcggg (78bp)</p>	<p>Cgl1169 promoter is the promoter of the Ncgl1122 gene in the following genomic context.</p>	short chain fatty acids transporter
Promoter: Cgl1141	<p>tt tcgatttcta aatgtcatat tggccgcttt ccaactatgt ttttgagcag gaggcgctt ttgatgtct taggggtggg tttctgggg ttacctagt ttttgaagg gtgaaatctc tatcgtctgg tgattggta ggaaatgctg tgatgcgagt ggtcttgggt gcgcgactcc atcaaaacca gacaagcgtg acaacggagc tcaatttct gagtttctgt cacgcctgtc tggttgcct cgaaaaggct gatttcacga ccaagatccc caaaagtctg acctctcaga atcgcttata agggccttc gtggccccgt ccatacaaac gcacattcgg aaaaatgaag ccctaaatc gccatacagc acctctctg aaatgcgcaa ccacggcaat</p>	<p>Cgl1141 promoter is the promoter of the Ncgl1096 gene in the following genomic context.</p>	K ⁺ transport flavoprotein

tcagccaatc
 tgcaataaaa
 aagtaaaact tatttagcgg
 cccgtgaaga
 tccccgaaaag
 gtcacaatga ccagccattt
 tgcctaaacc gtgtgctgga
 ttaaataggt gttaatatt
 aaaaactagc
 gagccctgaa ttaatctgtt
 aaggctctac
 atggacttca gttagttgag
 gctgaagtt ttgacatgta
 tgtctgtcc gagttcggc
 ccatccaggt
 ggtgcaccga
 ggtggattca tgggfaatcc
 ttaatcgagt
 gagggcccag
 tccaggggag
 cccgccgcat taagtgagtt
 cctgatggct tcgggtggag
 gatgtagaac
 tttttgaca tggctgtgg
 cacgcattgt ttcgtttgcc
 ctgccatgtt ggtgttttaa
 gtgaaggccg ccttttgccg
 aatgggtata
 ggaggcattc
 (852bp)

Promoter:
 Cgl0692

ggggtgct cctaaaaagc
 gaaggtcaaa
 aaacttgact ttattgaaaa
 caatttccat taagaagtgt
 acacttgctg
 cgactctatt gaaaatgatt
 cccaaaagga
 ggctttcac
 (118bp)

Cgl0692 promoter is the promoter of Ncgl0662 gene in the following genomic context.



G3E family GTPase

The PCR and cloning result of the promoters Cgl1538, Cgl1169, Cgl1141 and Cgl0692

The promoter of Cgl1538, Cgl1169 and Cgl1141 and Cgl0692 were PCR from the genome of *C. glutamicum* using Gold Star T6 Super PCR Mix (1.1X)TSE101, and HpaI/BamHI restriction endonuclease digest Cgl1538 PCR fragment and HpaI/EcoRI digest Cgl1169, Cgl1141 and Cgl0692

fragment respectively. The ligation vector was also digested with HpaI/BamHI or HpaI/EcoRI restriction endonuclease to remove the psod promoter from the vector plasmid. All the PCR promoter fragment and the cloning vector were run on the 1% agarose gel electrophoresis to check the size (Figure 3). All the ligation clones were confirmed by sequencing (Supplementary Information).

Extracting the constructed pXMJ(0692)-mCherry, pXMJ(Cgl1169)-mCherry, pXMJ(Cgl1141)-mCherry, and pXMJ (Cgl1538)-mCherry plasmids from the *E. coli* and run on the 1% gel to check the plasmid DNA size to verify the correct clones of the promoters.

After the promoter of Cgl0691, Cgl1169, Cgl1141 and Cgl1538 have been cloned into the original plasmid.

pXMJ-24nt-UTR-sod-mCherry, the constructed pXMJ(0692)-mCherry, pXMJ(Cgl1169)-mCherry, pXMJ(Cgl1141)-mCherry, and pXMJ (Cgl1538)-mCherry plasmids, which is designated as Cgl0692PATG, Cgl1169PATG, Cgl1141PATG, Cgl1538PATG plasmid in the gel respectively, was extracted to run on the 1% gel to check the plasmid DNA size to the correct clones of the promoters (Figure 4). J-12-CglPATG 0692, J-16-CglPATG 1169, AO6-11-CglPATG 1141 and AO3-CglPATG 1538 were selected for sequencing (Supplementary Information).

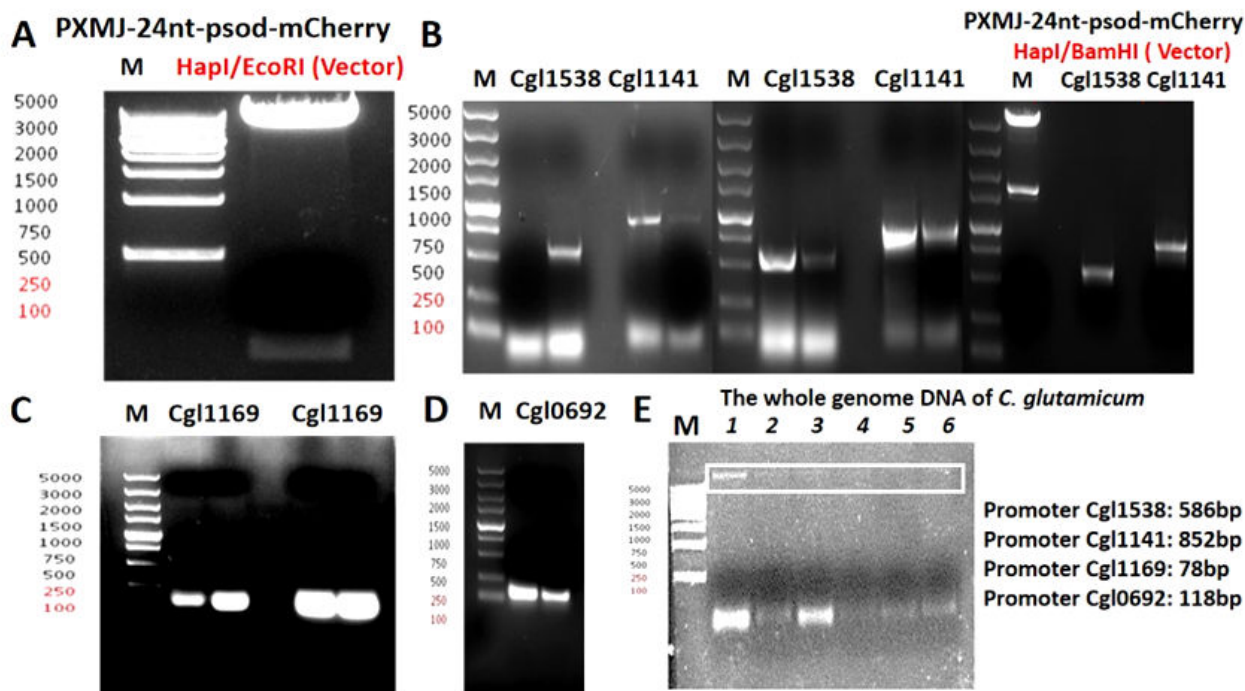


Figure 3) The candidate promoter of *Cgl1538*, *Cgl1169* and *Cgl1141* and *Cgl0692* were PCR from the whole genome of *C. glutamicum* and cloned at the corresponding restriction sites. (A) *HpaI/EcoRI* digest the pXMJ-psod-mCherry plasmid to gain the ligation vector (*HpaI/EcoRI*). (B) digest the pXMJ-psod-mCherry plasmid to gain the ligation vector (*HpaI/BamHI*). PCR the candidate promoters of *Cgl1538* and *Cgl1141* from the *C. glutamicum* genome, which were digested with *HpaI/BamHI* or *HpaI/EcoRI* restriction endonuclease. (C) PCR the promoters of *Cgl1169* from the *C. glutamicum* genome, which was digested with *HpaI/EcoRI* restriction endonuclease. (D) PCR the promoter of *Cgl0692* from the *C. glutamicum* genome, which was digested with *HpaI/EcoRI* restriction endonuclease. (E) Extraction the whole genome DNA of *C. glutamicum*.

Two promoters *Cgl1169* and *Cgl1141* are indeed in responsive to L-Ornithine or putrescine, but not *Cgl0692* promoter

The activity of the promoter characterized by selected random *C. glutamicum* clones was checked under the immunofluorescence value ratio OD535/OD600: Three random *C. glutamicum* clones which were electroporated *Cgl1538PATG*, *Cgl1169PATG*, *Cgl1141PATG*, *Cgl0692PATG* plasmids were selected from the LBHIS agar plate to check the activity of the promoter under the immunofluorescence value OD535/

OD600. The time-dependent response curves of wild-type *C. glutamicum* bacteria strain (ATCC13032), variants over expressed L-Ornithine (ER6937R42) and variants over expressed putrescine (16-17 CPVF-ALE) were presented in Figure 5. *Cgl1169* promoter is in responsive to L-Ornithine, and *Cgl1141* promoter is in responsive to putrescine indeed, because the final ratio of 1169PATG-ER6937R42/1169PATG-ATCC13032 (Final ratio: 1.15) and 1141PATG-16-17 CPVF-ALE/1141PATG-ATCC13032 (Final ratio: 1.20) are larger than 1.0, which is indicated that they have strong activity and were chosen for further analysis (Figures 5B, 5C and Table 4)

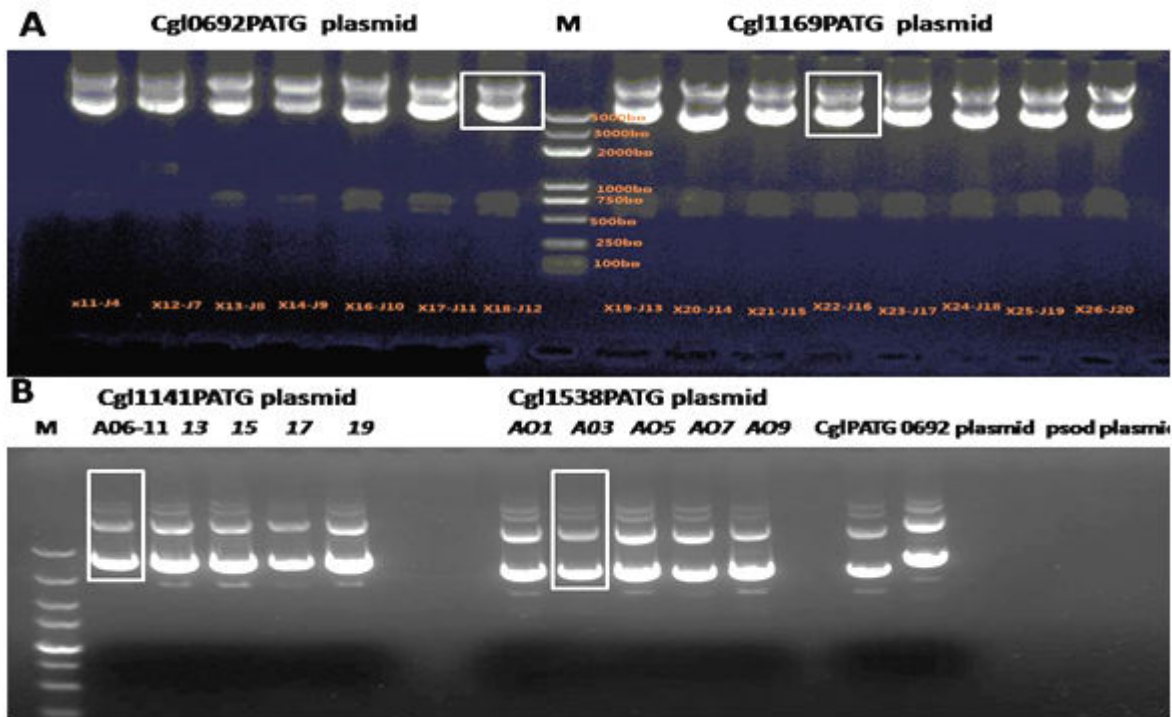


Figure 4) Extracting the *pXMJ(0692)-mCherry*, *pXMJ(Cgl1169)-mCherry*, *pXMJ(Cgl1141)-mCherry*, and *pXMJ (Cgl1538)-mCherry* plasmids from the *E. coli* and run on the 1% gel to check the plasmid DNA size to verified the correct clones of the promoters. The plasmid *pXMJ(0692,1169,1141,1538)-mCherry* is designated as *Cgl0692PATG*, *Cgl1169PATG*, *Cgl1141PATG*, *Cgl1538PATG* plasmid in the gel respectively. A) Extracting the *Cgl0692PATG*, *Cgl1169PATG* plasmids to check the plasmid DNA size to verified the correct clones of the promoters *Cgl0692* and *Cgl1169*. *J-12-CglPATG 0692* and *J-16-CglPATG 1169* were selected for sequencing. B) Extracting the *Cgl1141PATG*, *Cgl1538PATG* plasmids to check the plasmid DNA size to verified the correct clones of the promoters *Cgl1141* and *Cgl1538*. *AO6-11-CglPATG1141* and *AO3-CglPATG1538* were selected for sequencing.

TABLE 4 The final ratio of the promoters *Cgl1538*, *Cgl1169*, *Cgl1141* and *Cgl0692* characterized the promoter activity, all the assay were performed in triplicate. (3 random *C. glutamicum* clones were selected for each promoter activity test and averaged the detected data).

Promoter	Final ratio (ER6937R42/ATCC13032)	Final ratio (16-17 CPVF-ALE /ATCC13032)
<i>Cgl1538</i>	19411/22727=0.85	20882/22727=0.92
<i>Cgl1169</i>	6500/5643=1.15>1.0	3940/5634=0.70
<i>Cgl1141</i>	0	15040/12635=1.20>1.0
<i>Cgl0692</i>	7292/3115.6=2.343>1.0	0
Promoter	(ER6937R42 - ATCC13032)	(16-17 CPVF-ALE - ATCC13032)
<i>Cgl1538</i>	19411-22727=-3316	20882-22727=-1845
<i>Cgl1169</i>	6500-5643=857>0	3940-5643=-1703
<i>Cgl1141</i>	0	15040-12635=2405>0
<i>Cgl0692</i>	7292-3115.6=4176.4>0	0

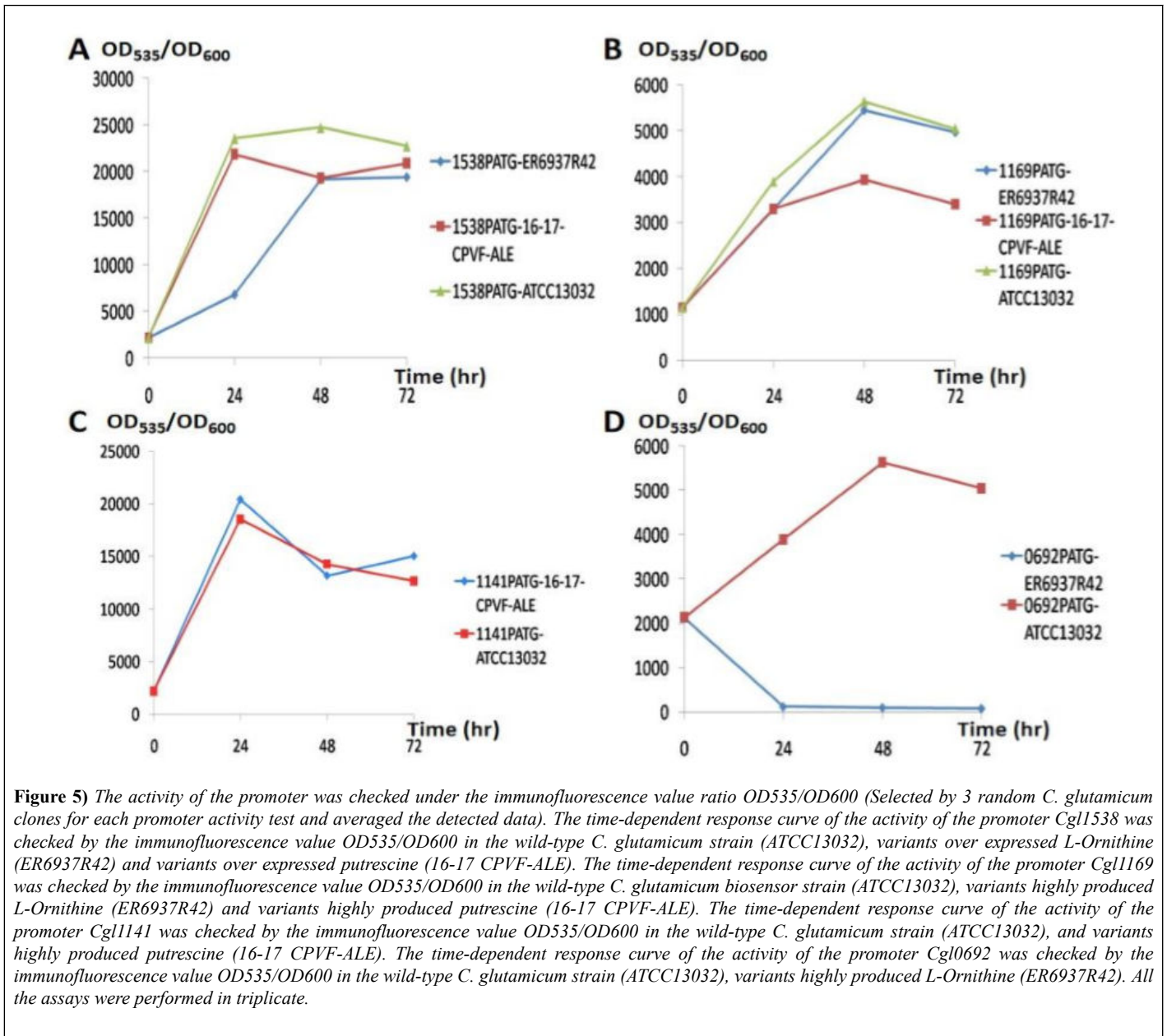


TABLE 5 The final ratio of the promoters *Cg11538*, *Cg1169*, *Cg1141* and *Cg10692* characterized the promoter activity, all the assay were performed in triplicate (Setting an initial bacterial density OD_{600} of 0.2; 3 bacterial culture samples for each promoter activity test and averaged the detected data).

Promoter	Final ratio (ER6937R42/ ATCC13032)	Final ratio (16-17 CPVF-ALE /ATCC13032)
<i>Cg11538</i>	21420/24333=0.88	17536/24333=0.721
<i>Cg1169</i>	17027/14679=1.161.0	13075/14679=0.891
<i>Cg1141</i>	14259/7479=1.911.0	
<i>Cg10692</i>	949.7/18430=0.051	

Promoter	(ER6937R42 - ATCC13032)	(16-17 CPVF-ALE - ATCC13032)
Cgl1538	21420-24333= -2913	17536-24333= -6797
Cgl1169	17027-14679= 23480	13075-14679= -1604
Cgl1141		14259-7479=67800
Cgl0692	949.7-18430= -17480.3	

The activity of the promoter was checked under the immunofluorescence value ratio OD535/OD600 (Setting an initial bacterial culture density OD600 of 0.2): The activity of the promoter has been checked under the ratio of OD535/OD600 by selected 3 random *C. glutamicum* clones (Checked at different time point of 0, 24, 48, 72 h,

Figure 5 and Table 4). Subsequently, the activity of the promoter was further confirmed by checking the immunofluorescence value ratio OD535/OD600 (Setting an initial bacterial culture density OD600 of 0.2) (Figure 6 and Table 5).

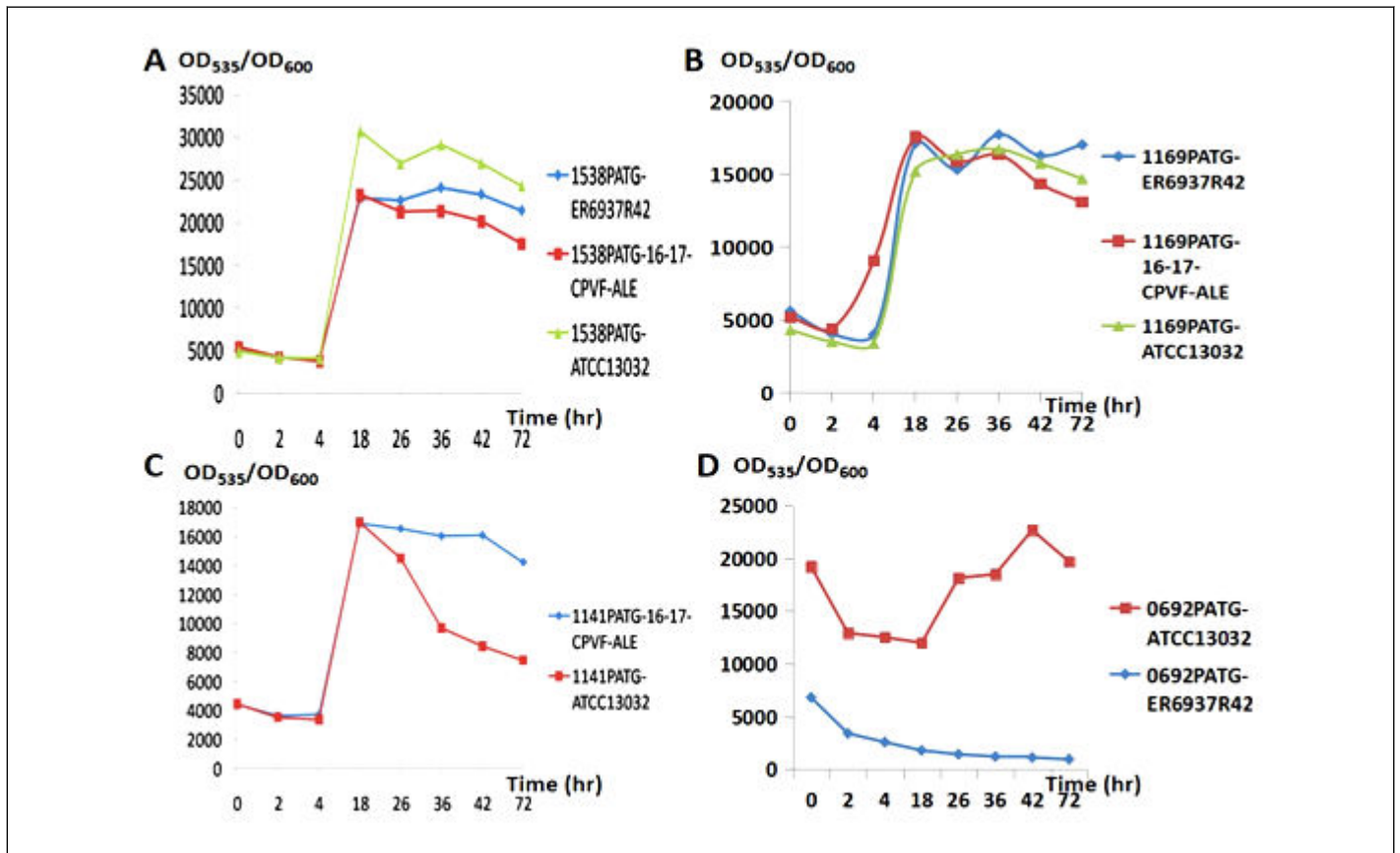


Figure 6) The activity of the promoter was characterized by the immunofluorescence value ratio OD535/OD600 (Setting an initial bacterial culture density OD600 of 0.2; 3 bacterial culture samples for each promoter activity test and averaged the detected data). The time-dependent response curve of the activity of the promoter Cgl1538 was characterized by the immunofluorescence value ratio OD535/OD600 with an initial setting bacterial density OD600 of 0.2 in the wild-type *C. glutamicum* strain (ATCC13032), variants over expressed L-Ornithine (ER6937R42) and variants over expressed putrescine (16-17 CPVF-ALE). The time-dependent response curve of the activity of the promoter Cgl1169 was characterized by the immunofluorescence value ratio OD535/OD600 with an initial setting bacterial density OD600 of 0.2 in the wild-type *C. glutamicum* strain (ATCC13032), variants over expressed L-Ornithine (ER6937R42) and variants over expressed putrescine (16-17 CPVF-ALE). The time-dependent response curve of the activity of the promoter Cgl1141 was characterized by the immunofluorescence value ratio OD535/OD600 with an initial setting bacterial density OD600 of 0.2 in the wild-type *C. glutamicum* strain (ATCC13032), variants over expressed putrescine (16-17 CPVF-ALE). The time-dependent response curve of the activity of the promoter Cgl0692 was characterized by the immunofluorescence value ratio OD535/OD600 with an initial setting bacterial density OD600 of 0.2 in the wild-type *C. glutamicum* strain (ATCC13032), variants over expressed L-Ornithine (ER6937R42). All the assays were performed in triplicate.

The quantitative analysis of the intracellular and extracellular concentration of L-Ornithine and putrescine: When the solution reach PH 1.0, L-Ornithine can react with the triketohydrindene hydrate to form

the red matter (Figure 7A), the quantity of which can be measured under fluorescence OD512 to determine the concentration of L-Ornithine (Figure 7B) [13,14].

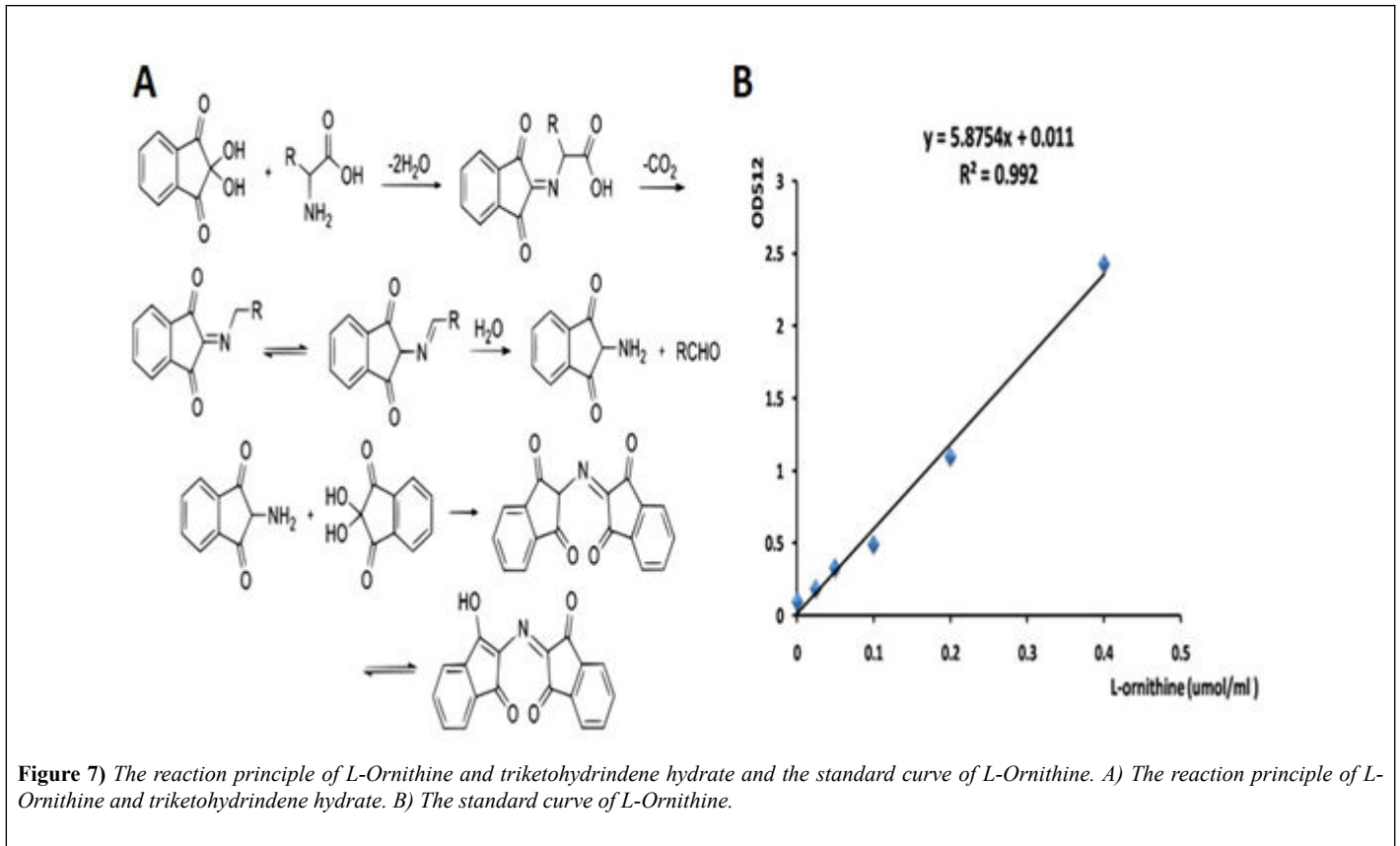


TABLE 6 The final intracellular and extracellular concentration of L-Ornithine

Extracellular	1169PATG (ER6937R42)	1169PATG (ATCC13032)
500/500	0.208	0.20
Concentration (µmol/mL)	0.033	0.032
Intracellular	1169PATG (ER6937R42)	1169PATG (ATCC13032)
500/500	1.165	0.469
Concentration (µmol/mL)	0.196	0.078
Concentration (µmol/mL)	(Intracellular) 0.196>0.033(Extracellular)	(Intracellular) 0.078>0.032(Extracellular)

So the standard curve of L-Ornithine can be made and the intracellular and extracellular concentration of L-Ornithine can be exactly determined by this approach. The assay was performed in triplicate (Table 6 and Table 7).

TABLE 7 The final intracellular and extracellular concentration of putrescine

Extracellular	1141PATG (16-17-CPVF-ALE)	1141 PATG (ATCC13032)
Concentration (μmol/mL)	0.670	0.370
Intracellular	1141PATG (16-17-CPVF-ALE)	1141 PATG (ATCC13032)
Concentration (μmol/mL)	0.330	0.150
Concentration (μmol/mL)	(Extracellular) 0.670>0.330(Intracellular)	(Extracellular) 0.370>0.150(Intracellular)

Construction of the L-Ornithine and putrescine inducible promoter-biosensor to promote biosynthesis of the valuable medicinal compound

Because the promoters of Cgl1169 and Cgl1141 are indeed in responsive to L-Ornithine or putrescine respectively, which were confirmed by above experiments, we will couple these promoters through brick DNA assemble technique to construct the chemical inducible promoter-biosensor to promote the biosynthesis of putrescine (Figure 1 and Figure 8A). On the one hand, once the L-Ornithine binding the transcription factor and the promoter of Cgl1169, it will trigger the allosteric response to activate the expression of L-Ornithine carboxylase which facilitates the rapid conversion L-Ornithine to synthesis of putrescine. This is “push” regulatory mode of L-Ornithine that promotes the synthesis of putrescine. On the other hand, excessive accumulation of putrescine will bind the

transcription factor and the promoter of Cgl1141 to active the expression of N-acetyl glutamic acid synthetase system ArgCJBD [15,16]. ArgCJBD will promote the conversion of glutamic acid to synthesis of L-Ornithine, supplement the consumption of L-Ornithine, which increases the intracellular level of L-Ornithine, and further promote the transformation to putrescine [15,16]. This is the remote “pull” regulatory mode of synthesis of putrescine. We expect to integrate both “push” and “pull” regulatory modes into a integrated genetic circuit to dynamically regulate synthesis of putrescine in *Corynebacterium glutamicum*. This “push” and “pull” genetic circuit will continuously promote the conversion and synthesis of from L-Ornithine to putrescine, due to the ArgCJBD route can supplement the consumption of L-Ornithine, which pushes forward the conversion of L-Ornithine to putrescine. It is predicted to present the kinetics curve with periodic change level (Figure 8B).

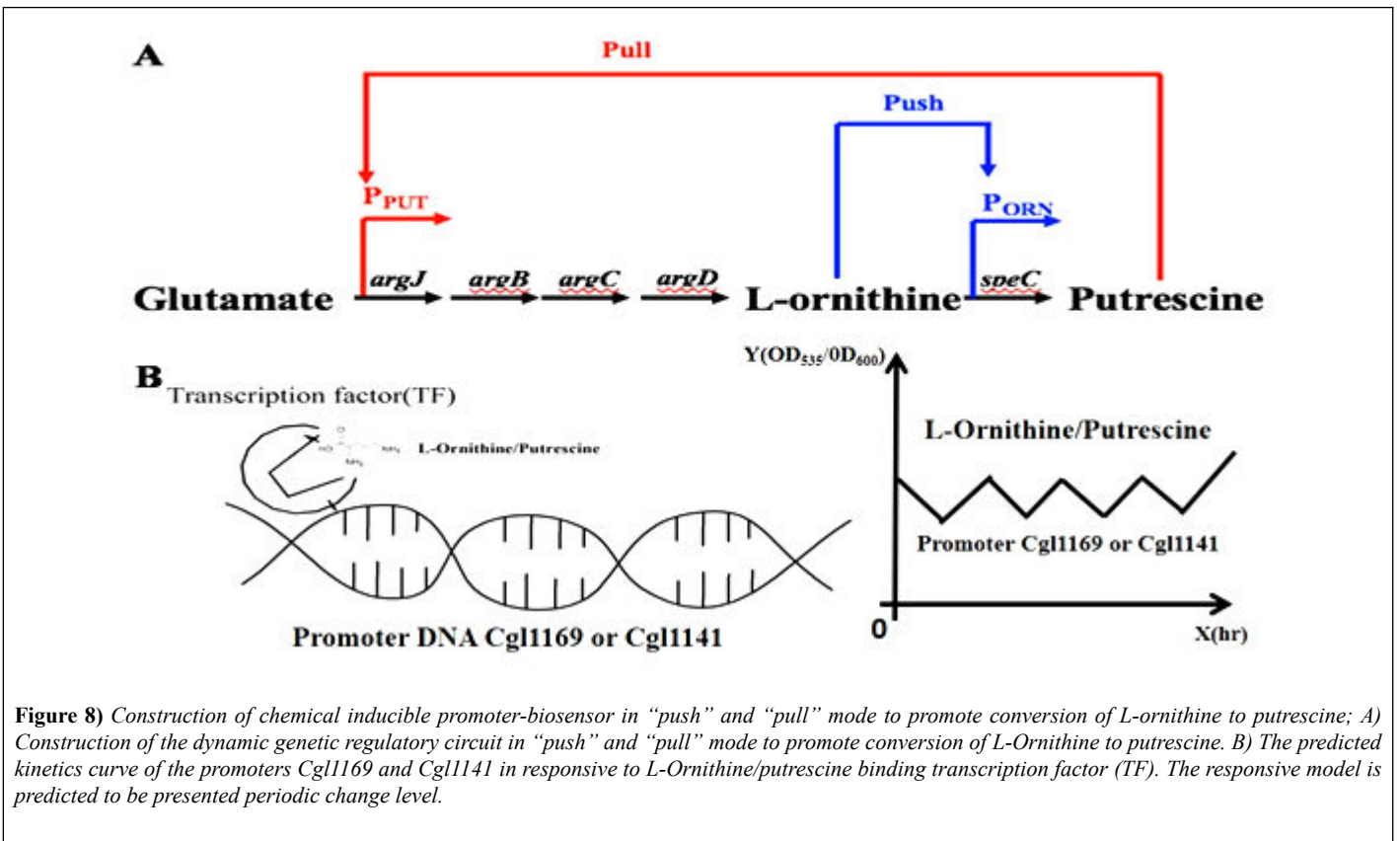


Figure 8) Construction of chemical inducible promoter-biosensor in “push” and “pull” mode to promote conversion of L-Ornithine to putrescine; A) Construction of the dynamic genetic regulatory circuit in “push” and “pull” mode to promote conversion of L-Ornithine to putrescine. B) The predicted kinetics curve of the promoters Cgl1169 and Cgl1141 in responsive to L-Ornithine/putrescine binding transcription factor (TF). The responsive model is predicted to be presented periodic change level.

DISCUSSION

In this paper, we have preliminarily constructed the chemical inducible promoter-biosensors which are in responsive to L-Ornithine or putrescine based on the comparative transcriptomics of *C. glutamicum*. Compared with wild type promoter-biosensor ATCC 13032, we find the promoter of Cgl1141 gene is indeed in responsive to putrescine and Cgl1169 is indeed in responsive to L-Ornithine, because the detected immunofluorescence value OD535/OD600 of Cgl1141 and Cgl1169 promoter in the mutant variant strain 16-17-CPVF-ALE/ER6937R42 is much higher than the wild type promoter-biosensor ATCC 13032 (with an initial setting bacterial culture density OD600 of 0.2) [Cgl1169Δ(ER6937R42-ATCC13032)

=17027-14679= 2348 > 0; Cgl1169 (ER6937R42/ ATCC13032) =17027/14679=1.16 > 1.0; Cgl1141Δ(16-17 CPVF-ALE - ATCC13032) =14259-7479=6780 > 0; Cgl1141 (16-17 CPVF-ALE / ATCC13032) =14259/7479=1.91 > 1.0] (Figure 6 and Table 5), the result was conformed to the result of selected random *C. glutamicum* clones (Figure 5 and Table 4). It is demonstrated that the Cgl1141 and Cgl1169 promoter in respective mutant variant strain in responsive to putrescine/ L-Ornithine is more sensitive than in the wild type strain ATCC 13032. However, we did not find the other two promoters Cgl0692 and Cgl1538 in responsive to L-Ornithine or putrescine, that is means, the chemicals L-Ornithine or putrescine may not favor the exogenous introduction of the promoters

Cgl0692 and Cgl1538. Based on this experimental results, we have searched the NCBI data base to find that the promoter Cgl0692 is connected with Cgl0987, so the continuous arrangement of the genes may not favor the exogenous introduction of the promoters Cgl0692, but this need further assay confirmation. Interestingly, it is found that the extracellular concentration of L-Ornithine in the culture medium is smaller than the intracellular concentration; however, the extracellular concentration of putrescine is larger than the intracellular concentration. It is speculated that the putrescine is secreted into the medium. The low intracellular concentration of putrescine may be more advantageous for the Cgl1141 promoter's responsive sensitivity and specificity with longer length, but L-Ornithine is highly accumulated intermediate chemicals which benefit the promoter activity to reduce the toxicity of L-Ornithine. Based on the correlation of the chemical concentration and the length of the promoter with the promoter's activity, we could further synthesize the promoters of *C. glutamicum* to enhance their activity (Figure 2).

It attracted our attention that we have found two sensitive promoters Cgl1141 and Cgl1169. Hypothetically, we could construct a genetic regulation modular which couples of the promoter Cgl1169 and Cgl1141 to create the chemical inducible promoter-biosensor with higher sensitivity to promote the biosynthesis of putrescine and L-Ornithine (Figure 8). This newly fabricated biosensor which utilizes the promoter with good properties will not only detect the toxic substances in medical care, but also maximum biosynthesis efficiency of medicinal compound for clinical applications [17-19].

CONCLUSION

The new high throughput screening method with the genetic construction approach has provided a good platform for high efficient biosynthesis of useful medicinal compound in microorganism. It will accelerate the development of the biosynthesis in medicine, pharmacology and petroleum fuel field.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interests.

ACKNOWLEDGEMENTS

We thank Prof. J.Z.L for helping with the molecular biology methods. We also thank Y.Z.F's wife X.N.J for providing the language editing and advice for this manuscript.

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