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

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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 pharmological 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 archaebacteria 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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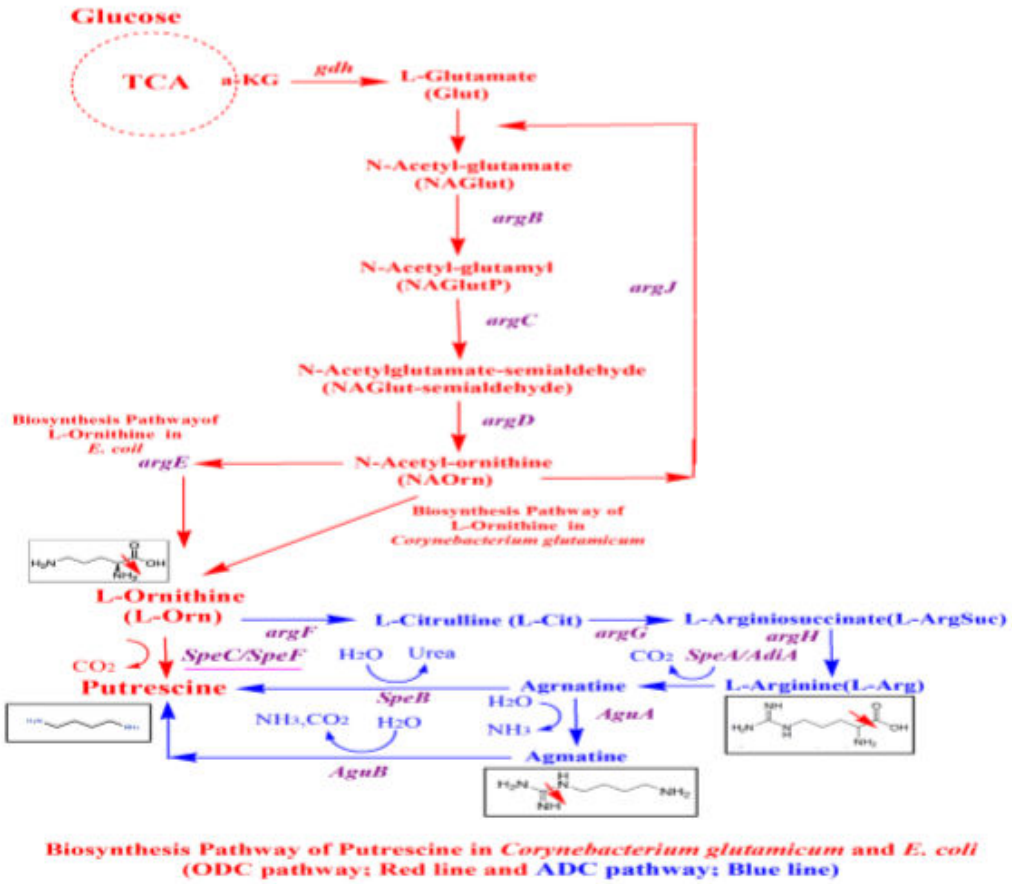


Figure 1) *Biosynthesis pathway of L-Ornithine and putrescine; Biosynthesis pathway of putrescine in C. glutamicum and E. coli (ODC pathway; Red line and ADC pathway: Blue line).*

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 immunoflurescence 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 isocaudarner 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' - ATTGTTAACAGATCTgtaaagttctaaagctttact-3'
Cgl1538(R-pATG)	R: 5' - CCGGGATCCcgtgacacctttacctgggt-3'
Cgl1169(F-pATG)	F: 5'- ATTGTTAACgcaaagcttattcgcgaaggc-3'
Cgl1169(R-pATG)	R: 5'-CCGGAATTCAAAAAGATCTcccgaaaatgcctgcgtagaa-3'
Cgl1141(F-pATG)	F: 5' - ATTGTTAACtttcgatttctaaatgtcata-3'
Cgl1141(R-pATG)	R: 5' -CCGGAATTCAAAAAGATCTgaatgcctcctatacccattc-3'
Cgl0692(F-pATG)	F: 5'- ATTGTTAACggggtgctcctaaaaagcgaa - 3'
Cgl0692(R-pATG)	R: 5' -CCGGAATTCAAAAAGATCTgtgaaagccctcctttggga-3'

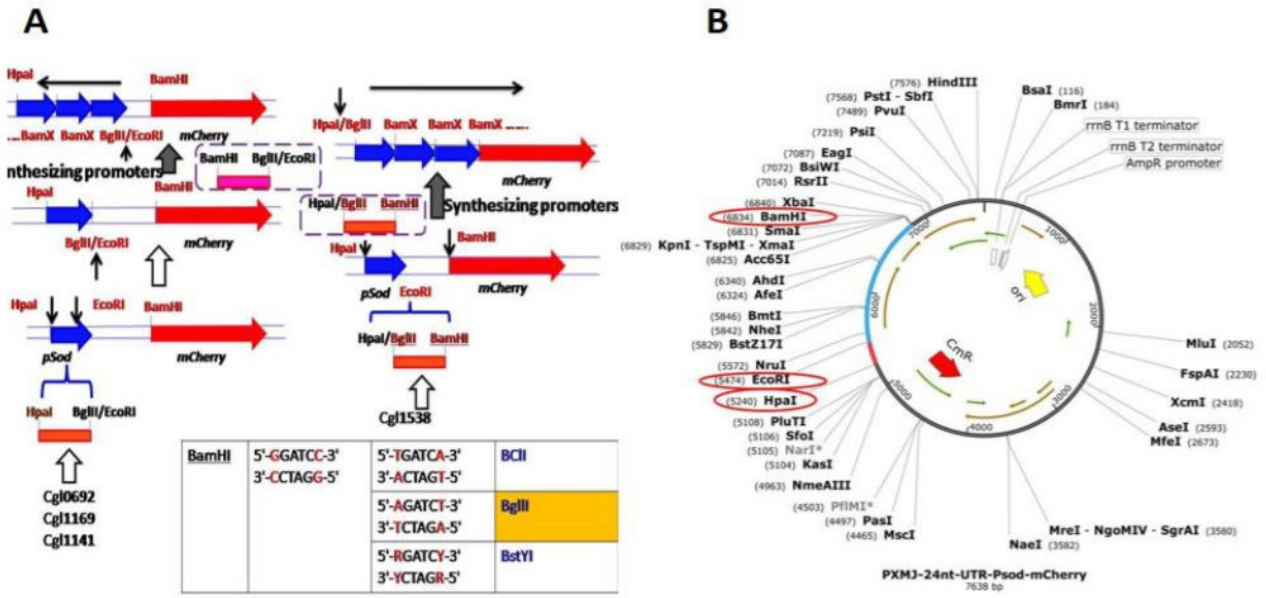


Figure 2) *Construction of the promoter -Immunofluorescence gene reporter system [pXMJ(Cgl1538)-mCherry, pXMJ (Cgl1169)-mCherry, pXMJ (Cgl1141)-mCherry and pXMJ(0692)-mCherry] and synthesizing the promoters of C. glutamicum by DNA brick assemble technique. A. The promoter of Cgl0692, Cgl1169 and Cgl1141 were synthesized through two steps, which added restriction endonuclease sites of HapI…….BglII-EcoRI and BamHI…….BglII-EcoRI. This constructing approach retained the part of DNA sequence from EcoRI to BamHI. The promoter of Cgl1538 was synthesized through one step, which added restriction endonuclease site of HapI-BglII……BamHI. This constructing approach removed part of DNA sequence from EcoRI to BamHI that made the backbone DNA sequence shorter. Isocaudarner of BglII and BamHI were used for all the constructs. B. The HapI, BglII and EcoRI restriction endonuclease sites in the original plasmid pXMJ-24nt-UTR-sod-mCherry.*

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 µL sterile water dissolves the plasmids of pXMJ (Cgl1538)-mCherry, pXMJ (Cgl1169)-mCherry, pXMJ (Cgl1141)-mCherry and pXMJ (Cgl0692)-mCherry. 100 µL competent cells of *C. glutamicum* mixed with 3 µL plasmid DNA in the pre-cold Gene Pulser®/MicroPulser™ 0.1 cm electroporation cuvette were cooled on ice for 10 min. Immediately, 800 µL 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 µL 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 µL supernatant was got to react with 500 µL 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 µL ddH_2O (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 µL supernatant was got to react with 500 µL 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 µm, 4.6 mm × 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

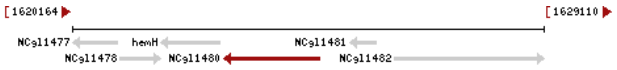
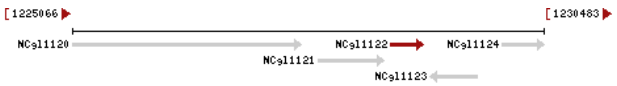
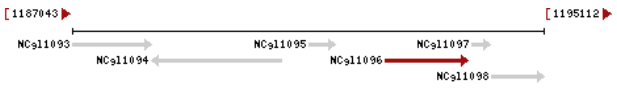
Search the genome library of *C. glutamicum* (NCBI) to find the promoter sequence of up-regulated genes. The promoter sequences of up-regulated genes (Table 2) were found in the genome library of *C. glutamicum* (NCBI). The promoter sequence and gene functions are listed in Table 3.

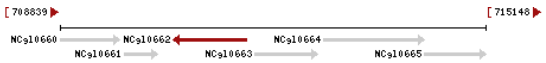
Corynebacterium glutamicum, a fast-growing and aerobic Gram-positive microorganism able to secrete large amounts of glutamate under suitable conditions, has a long history of use for the industrial production of various primary metabolites, including amino acids and nucleotides . The complete genome 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.

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-sod*mCherry*, which confers constitutive *mCherry* gene expression from cloned promoters.

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	gtaaag ttctaaagct ttacttatag cactttcggt aggtgtcgag aaaattccca aattgcggaaaattcacata ctttgtgcca caaagtgata aacatcacaa aatttcatga ctcagccattcatgcaggcc agaacatgca aaaccttgca cgctctggcc ttttgttatg taactgttgtgaaagatgta tcaaaagtta ccaaggtgac ttaatgttca gatatcgact catgggttgttttccaaaac ttgaatcaat ttgagtaaca gtagttatca agcgttaaac cttgaaacttccactctttt tactgatggg catttgggca aacggggaag cttgctggag atgcaataaatcggtgaatg gttggtgcaa tgaagaggag agtggcccat agtttccgct acggaacgcg gagtctttac gtttcaattc ttggcaagta tctagcgtca aggcttgata tgtgaagggattcggattga acaggagaac atgtggccaa tctagattca gttcgccctg gggtgcgggcagctcttcga cgtacccata cccaggtaaa aggtgtcacg (586bp)	Cgl1538 promoter is the promoter of Ncgl1480 gene in the following genomic context. 	Cell wall-associated hydrolase(invasion-associated proteins)
Promoter: Cgl1169	agcaaagct tattcgcgaa ggcatccaag gaacgtgggg attcattctt caattcccgt tctacgcagg cattttcggg (78bp)	Cgl1169 promoter is the promoter of the Ncgl1122 gene in the following genomic context. 	short chain fatty acids transporter
Promoter: Cgl1141	tt tcgatttcta aatgtcatat tggccgcttt ccactcatgt ttttgagcag gaggcggctt tttgatgtct taggggtggg tttctggggt tttacctagt ttttggaagg gtgaaatctc tatcgctggg tggattggta ggaaatgtcg tgatgcgagt ggtcttgggt gcgcgactcc atcaaaacca gacaagcgtg acaacggagc tcaattttct gagtttctgt cacgcctgtc tggtttgcct cgaaaaggct gatttcacga ccaagatccc caaaagttcg acctctcaga atcgcttata agggcctttc gtggccccgt ccatacaaac gcacattcgg aaaaatgaag cccttaaatc gccatacagc acctctctcg aaatgcgcaa ccacggcaat	Cgl1141 promoter is the promoter of the Ncgl1096 gene in the following genomic context. 	K+ transport flavoprotein

Promoter: Cgl0692	tcagccaatc tgcaataaaa aagtaaaact tatttagcgg cccgtgaaga tccccgaaag gtcacaatga ccagccattt tgcctaaacc gtgtgctgga ttaaataggt gttaaatatt aaaaactagc gagccctgaa ttaatctgtt aaggtcctac atggacttca gttagttgag gctgaagttt ttgacatgta tgtctcgtcc gagttcgccg ccatccaggt ggtgcaccga ggtggattca tggtgaatcc ttaatcgagt gaggccccag tccagggaag cccgccgcat taagtgagtt cctgatggct tcgggtggag gatgtagaac tttttgtaca tggtctgtgg cacgcattgt ttcgtttgcc ctgccatgtt ggtgttttaa gtgaaggccg ccttttgcgg aatgggtata ggaggcattc (852bp) ggggtgct cctaaaaagc gaaggtcaaa aaacttgact ttattgaaaa caatttccat taagaagtgt acacttgcgt cgactctatt gaaaatgatt cccaaaagga gggctttcac (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).

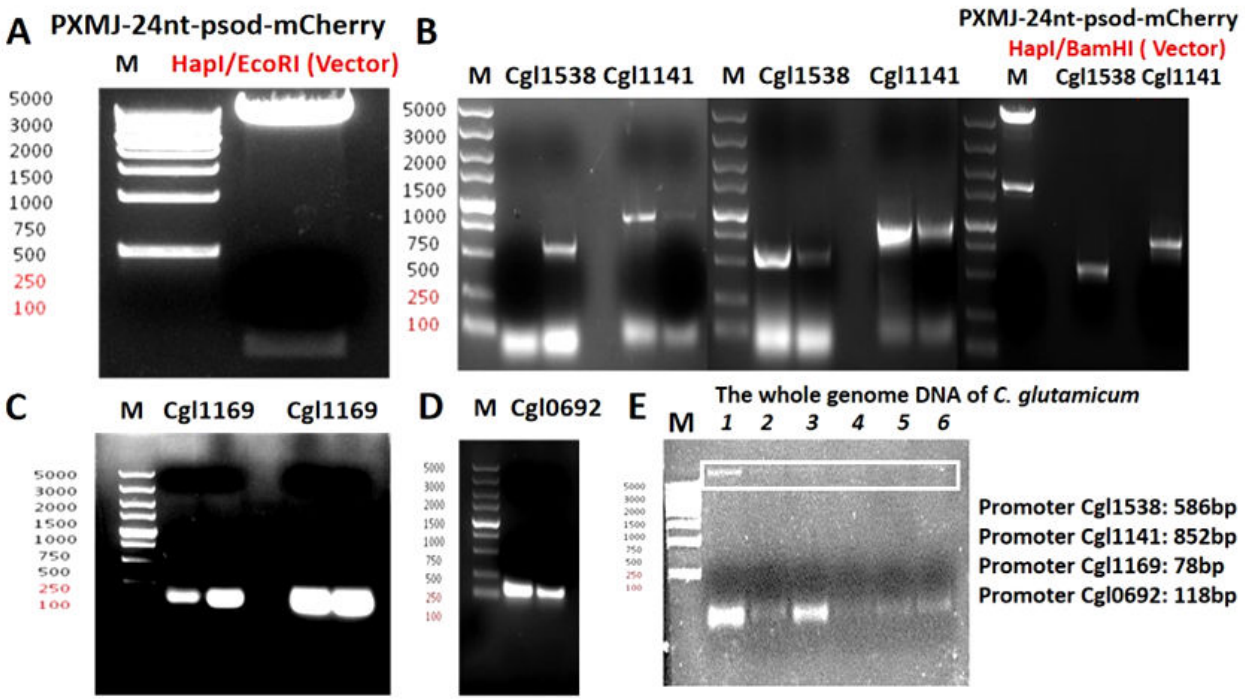


Figure 3) *The candidate promoter of Cgl1538, Cgl1169 and Cgl1141 and Cgl0692 were PCR from the whole genome of C. glutamicum and cloned at the corresponding restriction sites. (A) HpaI/EcoRI digest the pXMJ-psod-mCherry plasmid to gain the ligation vector (HpaI/EcoRI). (B) digest the pXMJ-psod-mCherry 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)

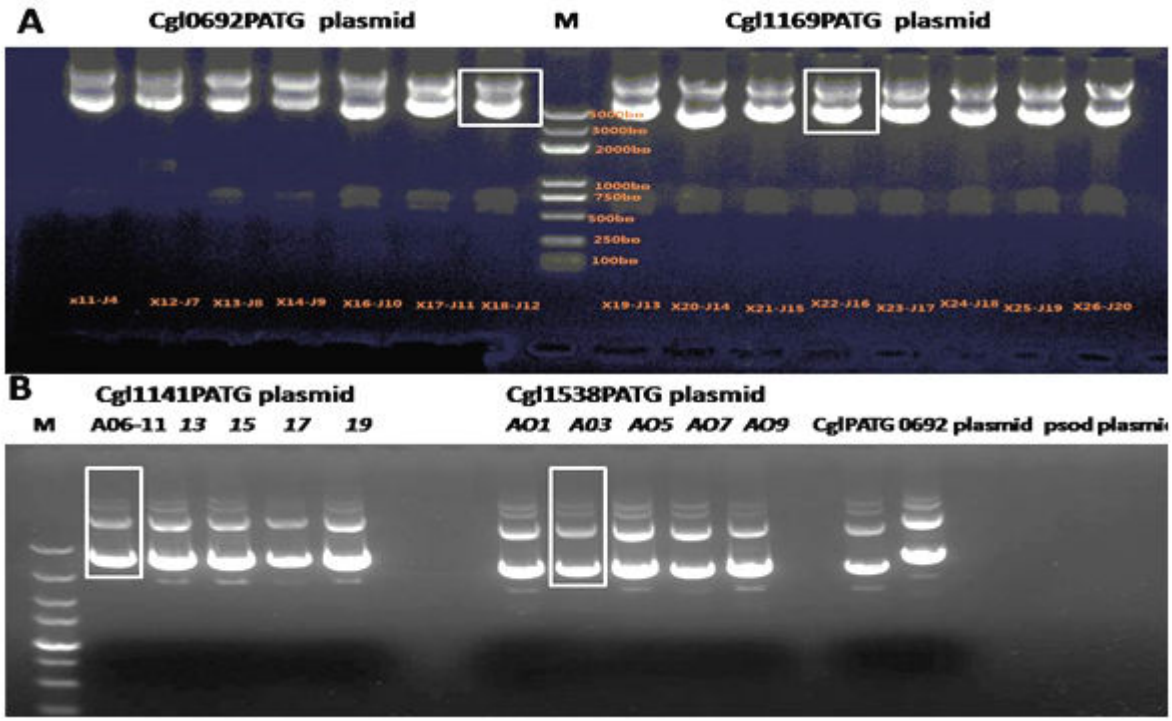


Figure 4) *Extracting the pXMJ(0692)-mCherry, pXMJ(Cgl1169)-mCherry, pXMJ(Cgl1141)-mCherry, and pXMJ (Cgl1538)-mCherry plasmids from the E. coli and run on the 1% gel to check the plasmid DNA size to verified the correct clones of the promoters. The plasmid pXMJ(0692,1169,1141,1538)-mCherry is designated 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)
Cgl1538	19411/22727=0.85	20882/22727=0.92
Cgl1169	6500/5643=1.15>1.0	3940/5634=0.70
Cgl1141	0	15040/12635=1.20>1.0
Cgl0692	7292/3115.6=2.343>1.0	0

Promoter	(ER6937R42 - ATCC13032)	(16-17 CPVF-ALE - ATCC13032)
Cgl1538	19411-22727= -3316	20882-22727= -1845
Cgl1169	6500-5643=857>0	3940-5643= -1703
Cgl1141	0	15040-12635=2405>0
Cgl0692	7292-3115.6=4176.4>0	0

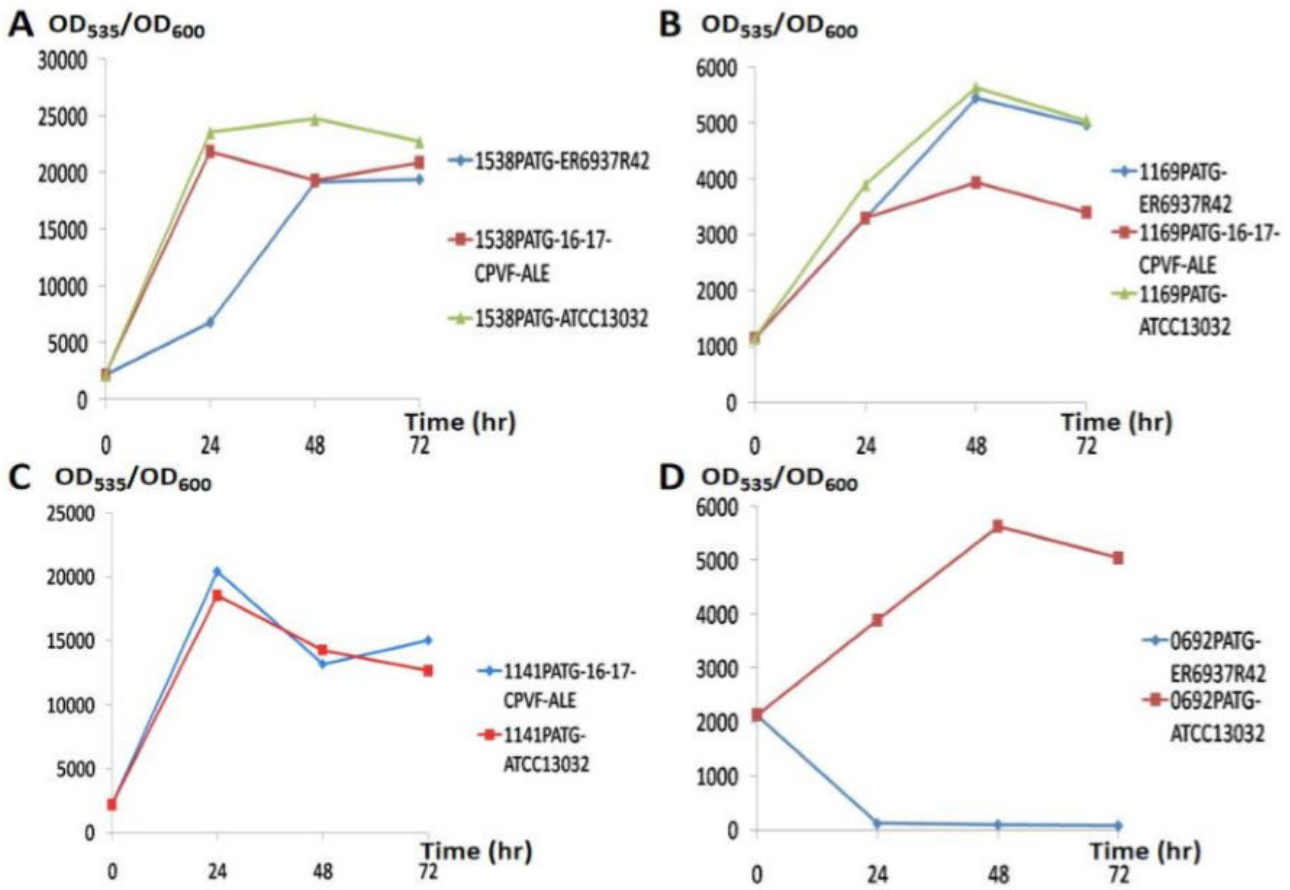


Figure 5) *The activity of the promoter was checked under the immunofluorescence value ratio OD535/OD600 (Selected by 3 random C. glutamicum clones for each promoter activity test and averaged the detected data). The time-dependent response curve of the activity of the promoter Cgl1538 was checked by the immunofluorescence value OD535/OD600 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 checked by the immunofluorescence value OD535/OD600 in the wild-type C. glutamicum biosensor strain (ATCC13032), variants highly produced L-Ornithine (ER6937R42) and variants highly produced putrescine (16-17 CPVF-ALE). The time-dependent response curve of the activity of the promoter Cgl1141 was checked by the immunofluorescence value OD535/OD600 in the wild-type C. glutamicum strain (ATCC13032), and variants highly produced putrescine (16-17 CPVF-ALE). The time-dependent response curve of the activity of the promoter Cgl0692 was checked by the immunofluorescence value OD535/OD600 in the wild-type C. glutamicum strain (ATCC13032), variants highly produced L-Ornithine (ER6937R42). All the assays were performed in triplicate.*

TABLE 5 The final ratio of the promoters Cgl1538, Cgl1169, Cgl1141 and Cgl0692 characterized the promoter activity, all the assay were performed in triplicate (Setting an initial bacterial density OD600 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)
Cgl1538	21420/24333=0.88	17536/24333=0.721
Cgl1169	17027/14679=1.161.0	13075/14679=0.891
Cgl1141		14259/7479=1.911.0
Cgl0692	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).

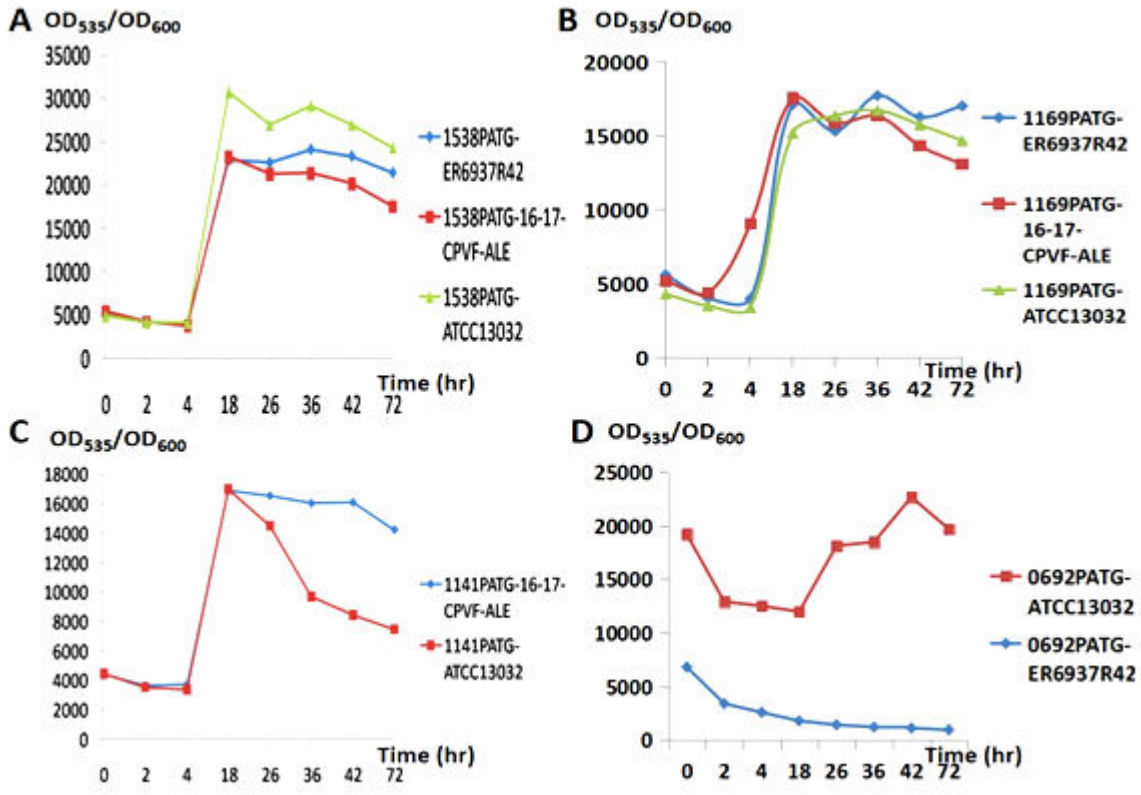


Figure 6) *The activity of the promoter was characterized by the immunofluorescence value ratio OD535/OD600 (Setting an initial bacterial culture density OD600 of 0.2; 3 bacterial culture samples for each promoter activity test and averaged the detected data). The time-dependent response curve of the 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].

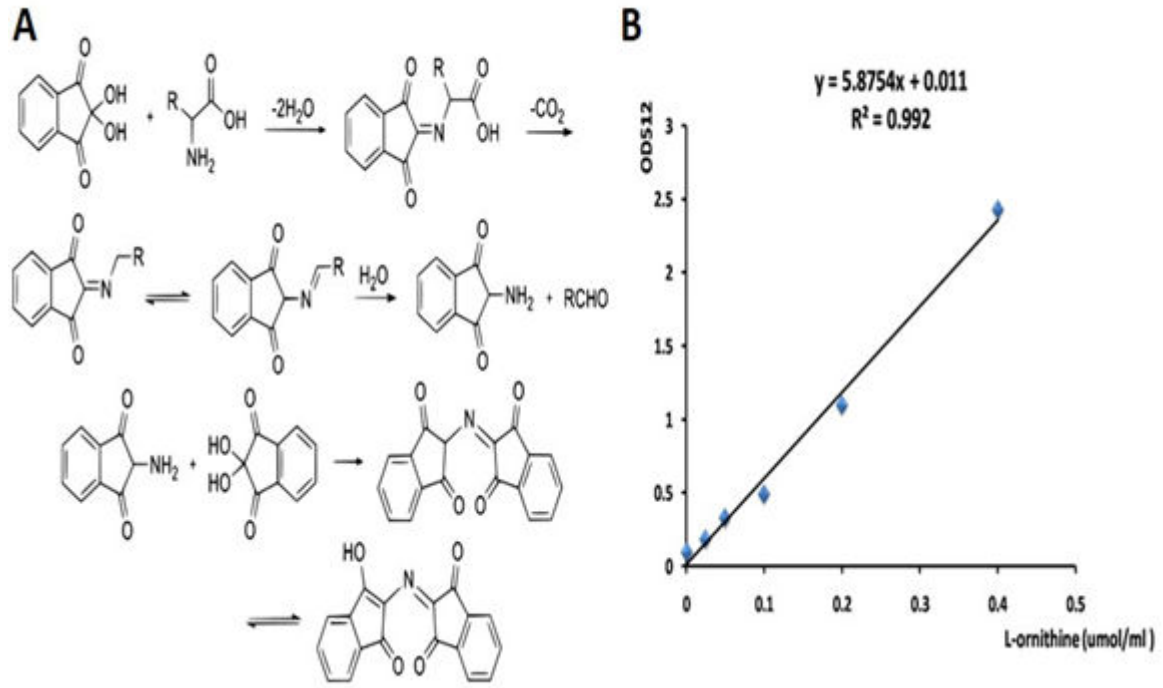


Figure 7) *The reaction principle of L-Ornithine and triketohydrindene hydrate and the standard curve of L-Ornithine. A) The reaction principle of L-Ornithine and triketohydrindene hydrate. B) The standard curve of L-Ornithine.*

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 active 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).

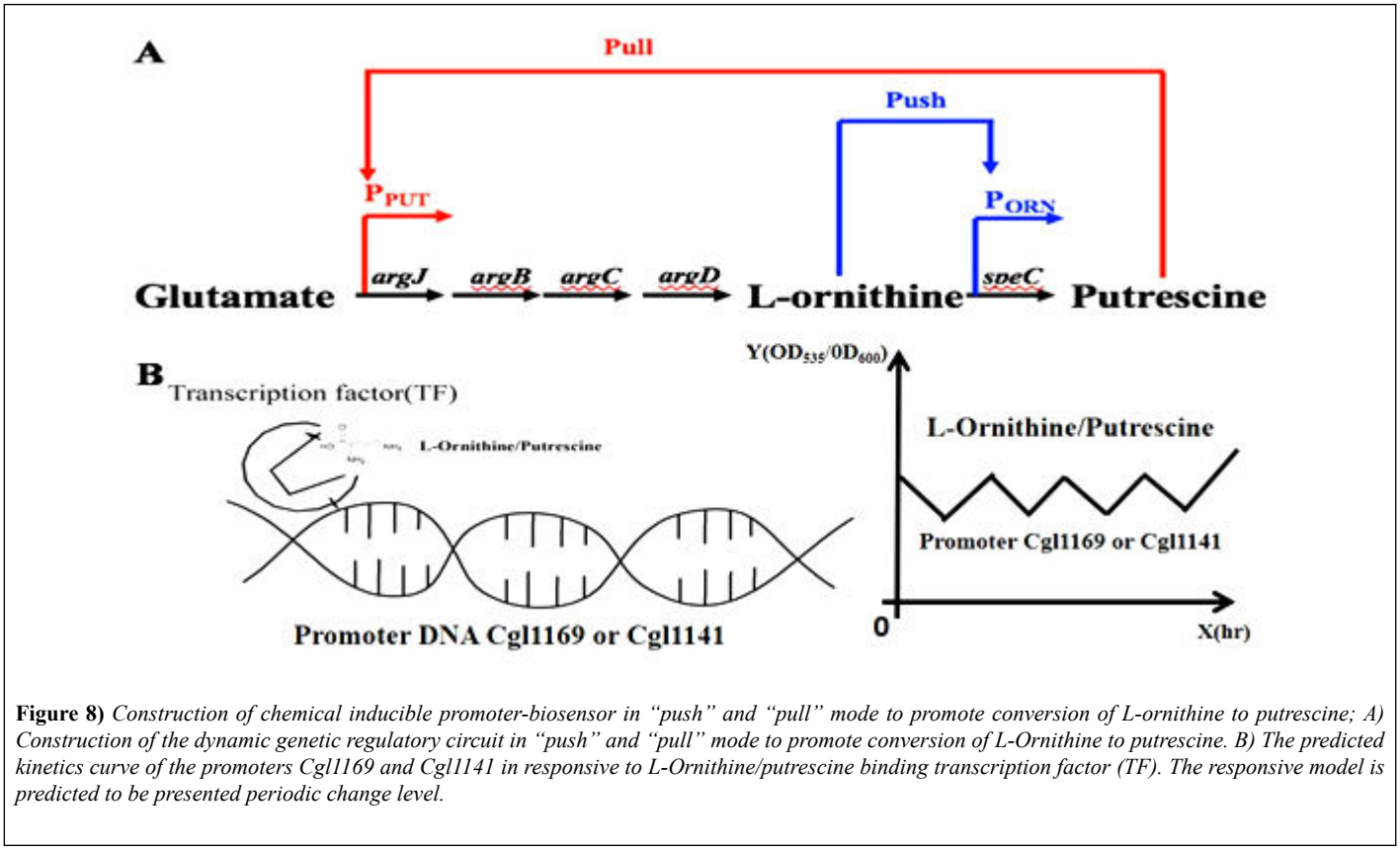


Figure 8) *Construction of chemical inducible promoter-biosensor in "push" and "pull" mode to promote conversion of L-ornithine to putrescine; A) Construction of the dynamic genetic regulatory circuit in "push" and "pull" mode to promote conversion of L-Ornithine to putrescine. B) The predicted kinetics 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) [Cgl1169△(ER6937R42-ATCC13032) =17027-14679= 2348 ＞ 0; Cgl1169 (ER6937R42/ ATCC13032) =17027/14679=1.16 ＞ 1.0; Cgl1141△(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.

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REFERENCES

1. Carothers WH. Linear polyamides and their production. US patent 2130523. 1938.
2. Nguyen A, Schneider J, Wendisch V. Identification of engineering targets for improving putrescine production by Corynebacteriumglutamicum. New Biotechnology. 2014;31:S166
3. Kurihara S, Oda S, Tsubio Y et al. γ-Glutamylputrescine synthetase in the putrescine utilization pathway of Escherichia coli K-12. J Biol Chem. 2008;283:19981-90.
4. Jiang L, Zhang Y, Li Z, et al. Metabolic engineering of Corynebacterium glutamicum for increasing the production of L-Ornithine by increasing NADPH availability. J Ind Microbiol Biot. 2013;40:1143-51.
5. Nguyen AQD, Scheneider J, Wendisch VF. Elimination of polyamine N-acetylation and regulatory engineering improved putrescine production by Corynebacterium glutamicum. J Biotechnol. 2015;201:75-85.
6. Nguyen AQD, Schneider J, Reddy GK, et al. Fermentative production of the diamine putrescine system metabolic engineering of Corynebacterium glutamicum. Metabolites. 2015;5:211-31.
7. Schneider J, Eberhardt D, Wendisch VF. Improving putrescine production by Corynebacterium glutamicum by fine-tuning Ornithine transcarbamoylase activity using a plasmid addiction system. Appl Microbiol Biotechnol. 2012;95:169-78.
8. Schneider J, Wendisch VF. Putrescine production by engineering Corynebacterium glutamicum. Appl Microbiol Biotechnol. 2010;88:859-68.
9. Inui M, Suda M, Okino S, et al. Transcriptional profiling of Corynebacterium glutamicum metabolism during organic acid production under oxygen deprivation conditions. Microbiology. 2007;153:2491-504.
10. Yukawa H, Omumasaba CA, Nonaka H et al. Comparative analysis of the Corynebacterium glutamicum group and complete genome sequence of strain R. Microbiology. 2007;153:1042-58.
11. Carneiro S, Villasboas S, Ferreira EC, et al. A comparative proteome analysis of Escherichia coli ΔrelA mutant cells. Front Bioeng Biotechnol. 2016;4:78.
12. Lin FM, Qiao B, Yuan YJ. Comparative proteomic of analysis of tolerance and adaptation of ethanologenic Saccharomyces cerevisiae to furfural, a lignocellulosic inihibitory compound. Appl Environ Microbiol. 2009;75:3765-76.
13. Yang M, Wang G, Wang S, et al. Quantitative analysis of 23-hydroxybetulinic acid in mouse plasma using electrospray liquid chromatography/mass spectrometry. Rapid Communications in Mass Spectrometry. 2005;19:1619-23.
14. Lee YJ, Cho JY. Genetic manipulation of a primary metabolic pathway for L-ornithine production in Escherichia coli. Biotechnol Lett. 2006;28:1849-56.
15. Ikeda M, Mitsuhashi S, Tanaka K, et al. Reengineering of a Corynebacterium glutamicum L-arginine and L-citrulline producer. Appl Envirion Microbiol. 2009;75:1635-41.
16. Makarova KS, Mironov AA, Gelfand MS. Conservation of the binding site for the arginine repressor in all bacterial lineages. Genome Biol. 2001;2:1-9.
17. Park JS, Lee JY, Kim HJ, et al. The role of Corynebacterium glutamicum spiA gene in whcA-mediated oxidative stress generegulation. FEMS Microbiology Letters. 2012;331:63-9.
18. Vertes AA, Inui M, Yukawa H. Manipulating Corynebacterium, from individual genes to chromosomes. Appl Envirion Microbiol. 2005;71:7633-42.
19. Qi L, Haurwitz RE, Shao W, et al. RNA processing enables predictable programming of gene expression. Nat Biotechnol. 2012;30:1002-6.