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Identification, repair and characterization of a benzyl alcohol-inducible promoter for recombinant proteins overexpression in *Corynebacterium glutamicum*

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ABSTRACT

Corynebacterium glutamicum is an important industrial organism for the production of a variety of biological commodities. We discovered a promoter encoded by the gene *NCgl2319* in *C. glutamicum*, which could be induced by benzyl alcohol, could be used as an efficient tunable expression system. In initial attempts, this promoter failed to function in a recombinant expression system. This was remedied by extending the original genetic context of the promoter, generating a new version Pcat-B. The Pcat-B transcription initiation site, its critical active regions, and its effect of inducers were fully characterized resulting in tunable expression. This approach proved to be very efficient in producing a pharmaceutical protein, N-terminal pro-brain natriuretic peptide (NT-proBNP). Production of approximately 440.43 mg/L NT-proBNP was achieved with the Pcat-B expression system demonstrating its application for controllable pharmaceutical protein production in *C. glutamicum*.

1. Introduction

Corynebacterium glutamicum has been widely used for nearly 60 years as a microbial cell factory to produce many small molecular commodities [1]. A deeper understanding of this strain should enhance its potential for recombinant protein expression [2]. *C. glutamicum,* a non-pathogenic microorganism with no endotoxin expression, has some advantages over *Escherichia coli*, making *C. glutamicum* an excellent host for food and pharmaceutical production [3]. Engineered strains of *C. glutamicum* have been used to produce pharmaceutical and diagnostic proteins such as a single chain antibody fragment (scFv) and a Fab fragment [2,4]. This related research and development has provided valuable information such as growing conditions and cellular mechanism. As exemplified by the large number of industrial applications of the *E. coli* expression system, a controllable expression system giving a high yield of protein from *C. glutamicum* would be highly desirable.

Gene expression in bacteria is controlled by a series of interacting genetic elements including promoter, 5′ untranslated reading frame (UTR), and open reading frame (ORF). Promoters are the primary elements to regulate protein abundance through transcription in biological systems [5]. They are also the primary switch elements in turning on and off systems in protein expression, metabolic engineering, and synthetic biology. Many strong promoters have been discovered by endogenous recognition, mutagenesis or fully-randomized synthesis [6,7]. Those methods are generally high-throughput and can efficiently identify strong constitutive promoters. However, when a recombinant protein is constitutively expressed in cells, the expression load can adversely impact cell proliferation, leading to low density of production units [8]. A production mode having both a proliferation phase and a production phase is highly beneficial in industrial applications and can be achieved under the control of a regulatable expression system. While regulatable promoters can be coordinated with high-density fermentation, they do not always afford high product expression levels [9]. Therefore, it is often necessary to develop an inducible expression system with the dual-capability of a high yield and controllability.

Several inducible promoters have been developed for the

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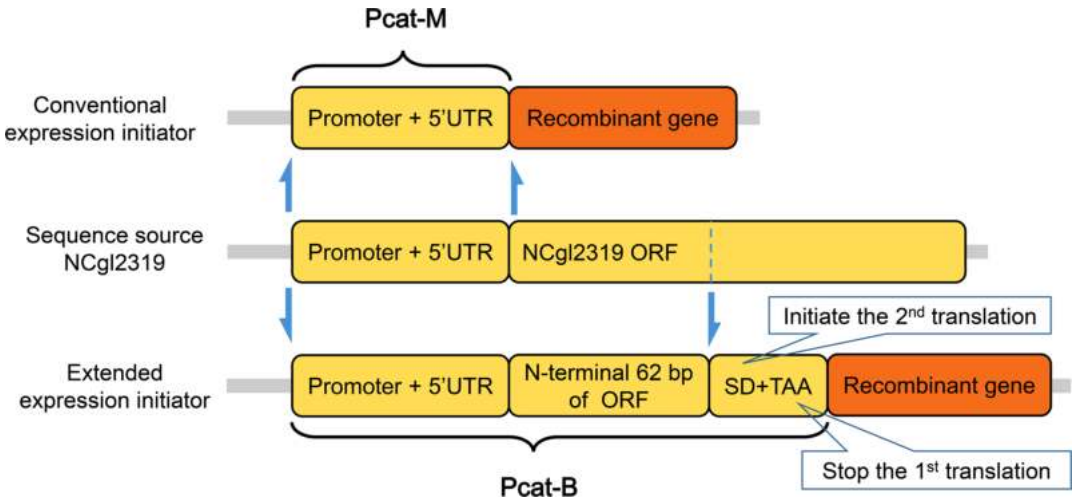

Fig. 1. Two strategies of using the NCgl2319 cis-regulatory element to initiate the expression of recombinant gene.

controllable protein expression in *C. glutamicum*. Some of these were derived from *E. coli* expression systems, such as P_{lacUV5} and P_{tac} from lactose operon, P_{trp} from *trp* operon, P_{araBAD} from *araC* gene, and P_R/P_L promoter from phage λ-*E. coli* system [6,10,11]. P_{lacUV5} and P_{araBAD} were developed years ago and lack sufficient data to evaluate their efficiency. Currently, pXMJ19 with tac promoter (P_{tac}) is commonly used as an expression vector for protein expression and metabolic engineering in *C. glutamicum*. The common inducer, isopropyl β-D-1-thiogalactopyranoside (IPTG), as an inducer for P_{tac} and P_R/P_L is not practical in industrial production because of its high cost and low permeability. In this work, we discovered a promoter from the genome of *C. glutamicum* that is induced by an inexpensive food additive, benzyl alcohol [12]. Although this benzyl alcohol-inducible promoter failed to work in the primary recombinant constructs, we examined whether it could be repaired using a 'context preservation' strategy. An optimized version of the promoter Pcat-B could be generated with significantly enhanced activity. The critical regions and inducer preference of Pcat-B were analyzed. The efficiency of Pcat-B expression vector was verified by enhanced expressing of green fluorescent protein (EGFP) and human protein N-terminal pro-brain natriuretic peptide precursor, NT-proBNP, a cardiac neurohormone used in diagnosing heart failure [13].

2. Materials and methods

2.1. Strains, plasmids and media

The bacterial strains and plasmids used in this study are listed in Supplementary Table 1. *C. glutamicum* CGMCC1.15647 was used as the host for protein expression. *E. coli* DH5α was used for DNA manipulation. A fully characterized *C. glutamicum* expression vector pXMJ19 with P_{tac} and a conserved Shine-Dalgarno (SD) sequence (AAAGGAGGACAAC) were used as a positive control [14,15]. A pXMJ19 backbone was applied in the construction of new expression vectors because of its capability of replicating in both *E. coli* and *C. glutamicum*. *C. glutamicum* was grown in LBB broth (Lysogeny broth (LB) medium added with 1% (w/v) brain heart infusion).

2.2. Identification of a benzyl alcohol-inducible gene in C. Glutamicum

C. glutamicum was grown overnight in 5 mL LBB medium at 30 °C, then inoculated at 1: 100 (v/v) into two Erlenmeyer culture flasks (no baffles) containing 50 mL LBB medium, one of which contained 10 mM benzyl alcohol. Cells pellets from 1 mL culture were harvested at 20 h after inoculation, followed by cell lysis in 1 mL phosphate buffered saline (PBS) and assayed by SDS-PAGE (5 μL sample was loaded on each lane) [16]. An extra strong protein band observed in the benzyl alcohol-induced sample was collected and analyzed with tandem mass spectrometry (MS/MS) in National Research Platforms (Jiangnan University) to identify the target protein. To verify these results, the 44-bp RBS sequence and the 85-bp N-terminal coding sequence of the identified gene was deleted from the genome of *C. glutamicum* by homologous recombination with pK18mobsacB (Supplementary Method 1) and the total proteins were assayed by SDS-PAGE.

2.3. Repair of natural promoter using 'Context preservation' strategy

First, the 500-bp cis-regulatory sequence upstream of the ORF of the benzyl alcohol-inducible gene was loaded into the Neural Network Promoter Prediction software (http://www.fruitfly.org/seq_tools/promoter.html) [16]. Prediction results supported our hypothesis that the putative promoter region was located within the upstream 500-bp sequence of ATG start codon. The 500-bp sequence containing promoter and 5′ UTR was amplified from the *C. glutamicum* genome as the conventional expression initiator (we called Pcat-M) for recombinant genes. When the promoter was found inactive in recombinant constructs, we adopted a 'context preservation' strategy to repair it by additionally amplifying the following 62-bp N-terminal coding sequence of the source gene followed by adding the conserved SD sequence and TAA stop codon (Fig. 1). The 5′ UTR with 60-bp N-terminal coding sequence should sufficiently reflect the effect of mRNA secondary structure and codon bias on translation initiation, playing a prominent role in the protein expression level [17]. We named the new version of expression initiator Pcat-B. Based on the bacterial polycistronic features, bacterial mRNA can carry multiple gene coding sequences that are separately translated [18]. When the repaired promoter was connected to a recombinant gene, a leading peptide and the recombinant gene could be successively translated. The actual transcription initiation site of the promoter was determined by the SMARTer® RACE 5′/3′ Kit (Takara).

2.4. Construction of expression vectors

The important genetic sequences used in this study are listed in Supplementary Data 1. All DNA manipulation procedures were carried out using standard protocols. Before the construction of expression vectors for Pcat-M and Pcat-B, P_{tac} and its regulatory gene *lacIq* from the *C. glutamicum* expression vector pXMJ19 were deleted for generating a intermediate shuttle vector p19−0 that could be propagated in both

E. coli and *C. glutamicum*. Genomic DNA was isolated using TIANamp Bacteria DNA Kit (TIANGEN BIOTECH, Beijing, China). The two promoters Pcat-M and Pcat-B were amplified from the genome of *C. glutamicum* CGMCC1.15647 and inserted into the vector p19−0 at the same location as P_{tac} in pXMJ19. The recombinant gene *egfp* or NT-proBNP was inserted into the expression vectors following either P_{tac}, Pcat-M or Pcat-B. *egfp*, a gene encoding fluorescent protein, was used to evaluate the expression activities for different promoters. NT-proBNP was another target gene in examining the promoter compatibility. The constructs included SUMO-NT-proBNP that carried an ubiquitin-like modifier (SUMO) tag to help protein fold into its active form.

A series of truncated promoters with different lengths of bp (i.e. 405 bp, 395 bp, 383 bp, 375 bp, 365 bp, 355 bp, 345 bp, 308 bp, 257 bp, 200 bp, 180 bp and 90 bp) were generated by cutting offtheir 5′ end to determine the authentic active region for the benzyl alcohol-inducible promoter in Pcat-B. The lengths were calculated from renewed N-terminal end to the beginning of coding sequence.

2.5. Protein extraction and analysis

C. glutamicum was inoculated into 100 mL fresh LBB medium at a ratio of 1:100 (v/v) and grown at 30 °C, then induced by 10 mM (default) benzyl alcohol or 1 mM IPTG when the OD_{600} reached to 0.1. After 20 h induction, cells of each sample with the amount equal to 1 mL of 10 OD_{600} were harvested by centrifugation and washed twice with PBS to remove extracellular proteins and other contaminants, then re-suspended in 1 mL PBS. Cell lysis was carried out using Ultrasonic breaker Vibra cell VCX500 (SONICS, USA).

The total protein concentration in the culture fluid was determined by Bradford's method with Bradford kit (Sangon Biotech, China). Samples normalized by total protein concentrations were loaded on 12 % SDS-PAGE gels.

2.6. Quantification of fluorescence intensity and transcription level

Intracellular expressions of EGFP in different constructs were observed through fluorescence microscopy. Cells grown for 24 h were harvested, washed and re-suspended followed by imaging the intra cellular fluorescence using a fluorescence microscope BX53 (Olympus Corporation, Japan). Fluorescence intensities were measured using a multimode plate reader (Tecan Infinite Pro 200, Switzerland) to quantify the expression levels of EGFP in *C. glutamicum*. The fluorescence intensities were normalized by cell optical density OD_{600} so that values indicated EGFP expression level of a single cellular unit.

The transcription levels of *egfp* were quantified by quantitative real time PCR (qRT-PCR) as described previously [19]. A $2^{-\Delta\Delta Ct}$ method was applied with 16S rRNA as the housekeeping gene. The level of Pcat-M construct in non-induced condition was defined as 1.0.

2.7. Quantification of SUMO-NT-proBNP

A sandwich two-step immunofluorescence assay with two types of antibodies specific to different epitopes of BNP was used to confirm and quantify the expression of SUMO-NT-proBNP [20]. The biotinylated capture antibodies, SUMO-NT-proBNP and horseradish peroxidase (HRP)-labeled detection antibodies were added onto microtiter plate sequentially. The HRP catalyzed luminol-H_2O_2-*p*-iodophenol (PIP) chemiluminescent (CL) system (Autobio, Zhengzhou, China) and LUmo Luminometer (Autobio) were used to quantify the levels of NT-proBNP. The recombinant NT-proBNP (Roche, Rotkreuz, Switzerland) was used as a standard to calculate the yield of NT-proBNP.

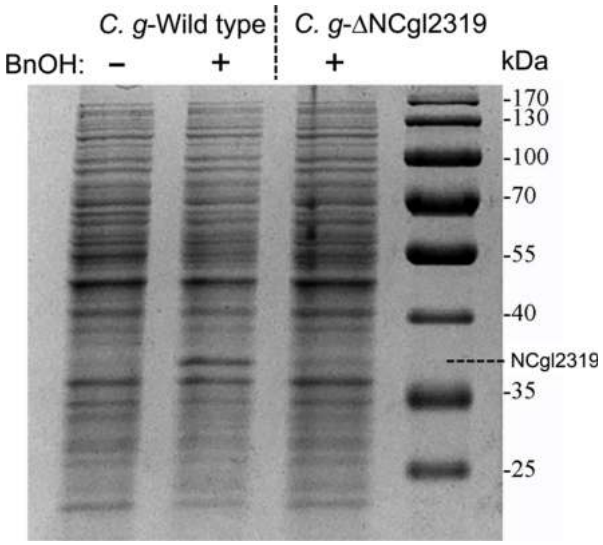

Fig. 2. Analysis of total proteins for the wild type *C. glutamicum* and *C. glutamicum* ΔNCgl2319. Cell samples were grown with or without 10 mM benzyl alcohol (BnOH), which are indicated by the symbol + and -.

3. Results and discussion

3.1. Discovery of a benzyl alcohol-inducible gene

Benzyl alcohol naturally occurs in plants, fruits and teas [21]. It can also be used as flavoring substance for foods and wine [12]. We found benzyl alcohol induced *C. glutamicum* to highly express an unknown protein. *C. glutamicum* was grown in LBB with or without 10 mM benzyl

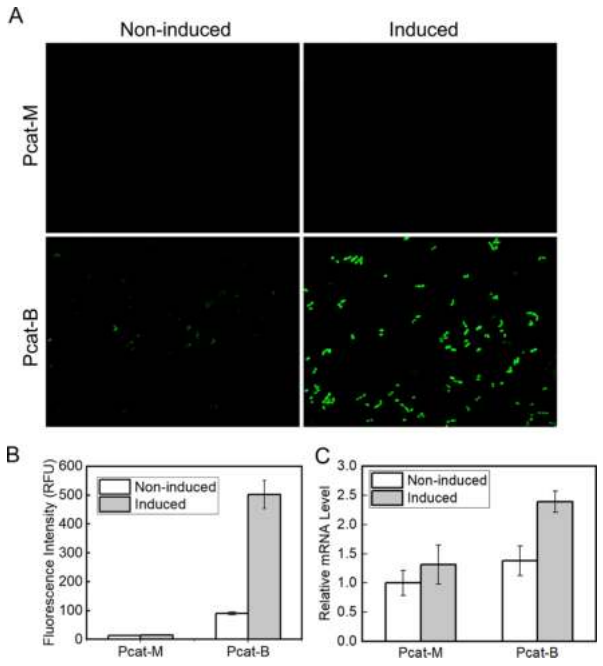

Fig. 3. Comparison of the EGFP expression between Pcat-M and Pcat-B constructs. Samples were either induced by benzyl alcohol or not. (A) Images of fluorescent microscopy for Pcat-M and Pcat-B constructs. (B) Quantification of the fluorescent intensity and (C) relative transcriptional level for the two constructs.

alcohol to identify the unknown protein. Cells were harvested and the total proteins of both samples were isolated for SDS-PAGE assay (Fig. 2). The results showed that with addition of benzyl alcohol in LBB, wild type of *C. glutamicum* cells highly expressed an unknown protein of molecular weight ~35 kDa. The protein band was extracted and analyzed by MS/MS. The protein was identified as catechol 1,2-dioxygenase (35 kDa, GenBank accession no. NCgl2319, also EFG81679.1) [22]. To confirm its identity, the gene NCgl2319 was deleted from the genome of *C. glutamicum* by homologous recombination (Supplementary Fig. 1), and a mutant *C. glutamicum* with ΔNCgl2319 was grown in LBB medium under the same condition with benzyl alcohol. SDS-PAGE (Fig. 2, Lane 3) of the total proteins from the mutant cell showed that the unknown protein band disappeared. Based on the above results, we concluded that this protein was expressed from gene NCgl2319 and its expression was regulated by benzyl alcohol. Thus, the promoter should be a benzyl alcohol-inducible promoter.

3.2. Two strategies for the utilization of cis-regulatory sequence from NCgl2319

The Neural Network Promoter Prediction software (version 2.2) predicted that the promoter region of NCgl2319 was within the 500-bp cis-regulatory sequence upstream of the start codon ATG. Next we tried to utilize this 500-bp cis-regulatory sequence (i.e., promoter-5′ UTR named Pcat-M) to initiate the expression of recombinant genes.

Fluorescent protein EGFP was used as reporter to indicate the activities of cis-regulatory elements. In our initial trial on the 500-bp cis-regulatory element, Pcat-M was directly connected to the reporter gene *egfp*. Surprisingly, no fluorescence was detected in cells, which suggests that EGFP was barely expressed under the control of the promoter-5′ UTR region (Fig. 3). Since high expression of NCgl2319 was observed, we believe the cis-regulatory sequence of NCgl2319 has a strong activity.

In bacteria, the recombinant gene expression sequence can be built in polycistronic structure so that an mRNA carries multiple genes and translates them separately through each SD sequences upstream of the corresponding gene [23]. Independently translating a short peptide gene before the target gene may increase the yield of target protein, due to the change of initial translation efficiency [19]. Intrinsic helicase activity of ribosomes could be utilized to minimize inhibitory RNA structure of target protein sequence [24]. It inspired us to utilize the discovered promoters in a new sequence structure. We extended the range of cis-regulatory sequence cloned from NCgl2319 by cloning the promoter-5′ UTR sequence and the following 62-bp N-terminal open reading frame of NCgl2319. An SD sequence and a stop codon TAA were added behind it for ending the first translation and starting the second (Fig. 1). This extended functional sequence was named as Pcat-B and connected to the target gene *egfp*. In this design, a short peptide of the N-terminal NCgl2319 is first translated and released, then the target gene *egfp* is translated.

The two cis-regulatory elements Pcat-M and Pcat-B have significant differences in modulating the expression of EGFP. Bright fluorescence was observed from Pcat-B constructs growing with 10 mM benzyl alcohol inducer (Fig. 3A). The quantification of fluorescence intensities of cells from different constructs confirmed our design: Pcat-M failed to initiate the expression of EGFP with or without the inducer, while significant increase of expression was achieved in Pcat-B form (Fig. 3B). In LBB medium supplemented with benzyl alcohol, the activity of Pcat-B was 34-times higher than Pcat-M, which fully demonstrated the effectiveness of the extended form of this promoter in *C. glutamicum*. Although the Pcat-B construct had some background expression, the induced expression level was 6.5-fold higher than the non-induced. In most cases, this background level was not a heavy load in cell culture. Further studies were conducted around transcriptional regulators to achieve highly-regulated expression (easily be engineered or overexpressed) [25].

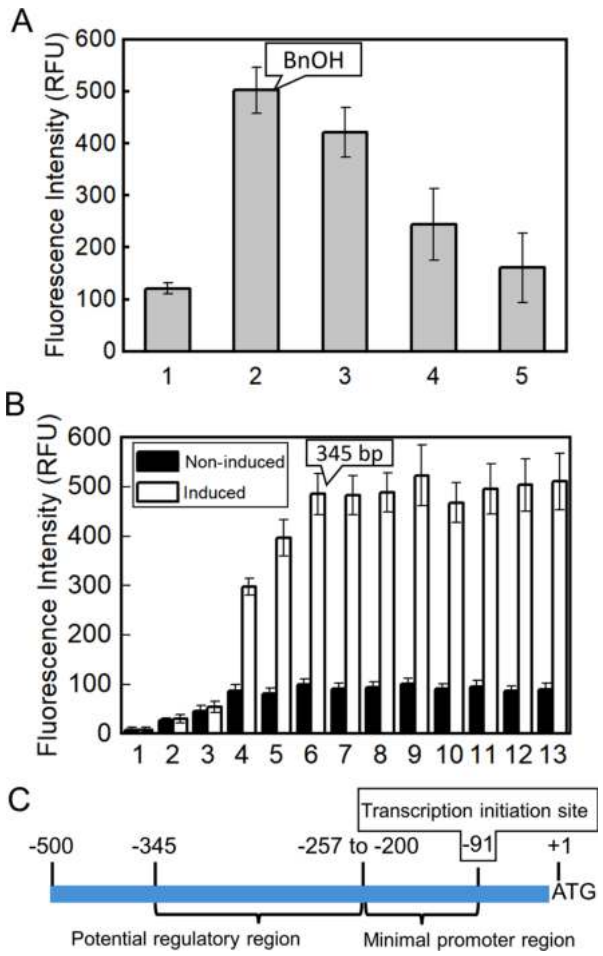

Fig. 4. EGFP expression levels in either different constructs or conditions. (A) The EGFP expression levels in Pcat-B construct induced by 10 mM benzyl alcohol or its metabolic intermediates. 1. No inducer control; 2. benzyl alcohol, 3. benzaldehyde; 4. benzoate; 5. catechol. (B) Activities of the truncated Pcat-B promoters in different lengths. 1-13: 98 bp, 180 bp, 200 bp, 257 bp, 308 bp, 345 bp, 355 bp, 365 bp, 375 bp, 385 bp, 395 bp, 405 bp, 500 bp. The lengths were calculated from promoter N-terminal end to the start codon ATG of ORF. (C) Schematic of the promoter from NCgl2319.

RT-PCR experiment was carried out to compare the transcription levels to investigate the mechanism of extended form Pcat-B giving higher expression of EGFP than Pcat-M. The level of transcription in Pcat-B construct was about 1.8-times higher than the level of Pcat-M construct (Fig. 3C). Initially, we hypothesized that the additional NCgl2319 N-terminal coding sequence together with promoter-5′ UTR were recognized and bound by transcription initiation complex as long as the promoter was close enough to the start codon ATG. We found the transcription initiation site was -91 bp away from ATG, and transcription initiation complex could not cover such a long sequence during recognition by 5′RACE assay. This seems an unlikely reason for the slight increase of transcription level in Pcat-B constructs. It has been reported that yeast utilized a leading peptide sequence to alter ribosome occupancy and then RNA stability, which might be an explanation of our observation on RNA disparity [26]. This alone does not explain a 34-fold difference in expression level. Since the two constructs had the same plasmid backbone (we assume the plasmid copy numbers were similar) and growing condition, we hypothesized that the N-terminal ORF sequence could influence the translation efficiency of EGFP [5]. RNA secondary structure of the translation initiation region of for Pcat-M and

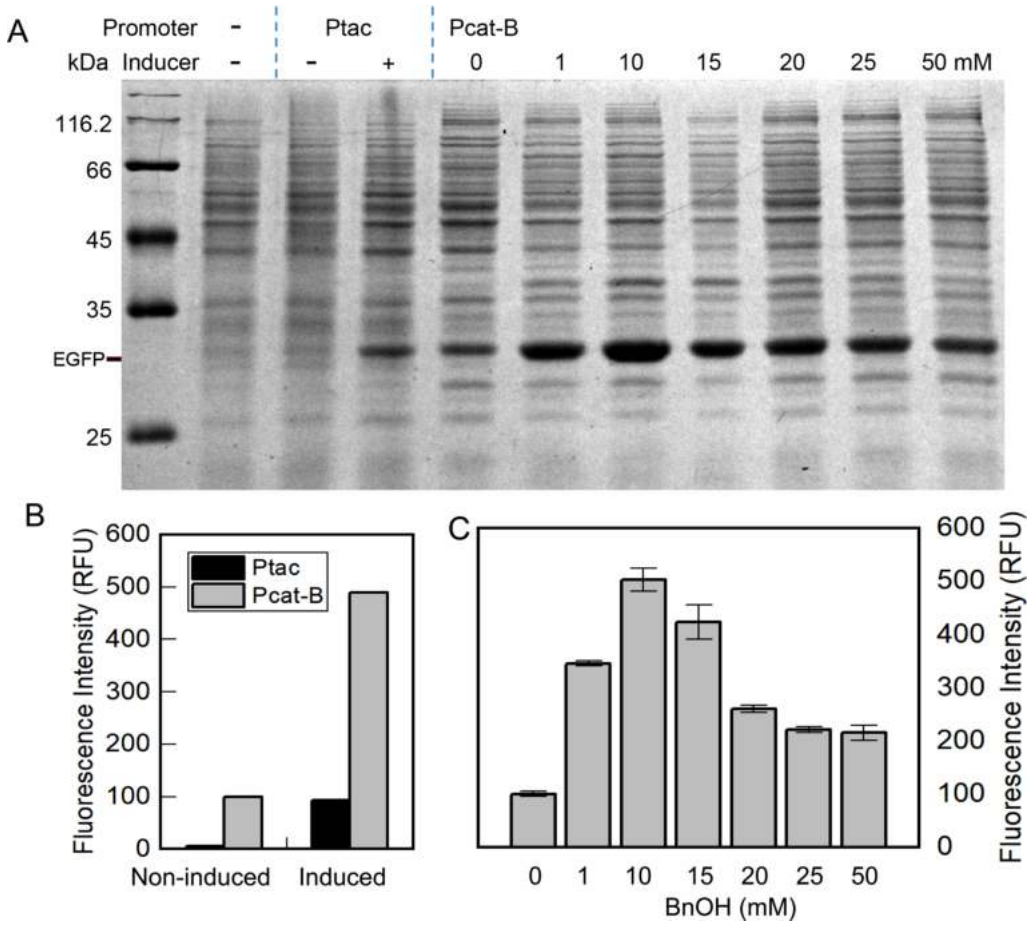

Fig. 5. Optimization of the inducing concentration for Pcat-B construct. (A) SDS-PAGE analysis and (C) fluorescent quantification of the EGFP expression level with different concentrations of benzyl alcohol. 1-7: 0, 1 mM, 10 mM, 15 mM, 20 mM, 25 mM, and 50 mM. (B) Comparison of expression activities between Pcat-B and P_{tac}.

Pcat-B constructs were predicted in RNAfold WebServer (http://rna.tbi.univie.ac.at//cgi-bin/RNAWebSuite/RNAfold.cgi) [27]. Pcat-M translation initiation region has a more stable mRNA structure (minimum free energy: -46.80 kcal/mol) than that of NCgl2319 (minimum free energy: -28.80 kcal/mol), which may inhibit ribosome binding and initiation (Supplementary Fig. 4). If an endogenous gene was observed highly expressed but its promoter-5′TUR failed to work, it may be due to the inhibitory mRNA secondary structure and it is worth additionally preserving the original N-terminal sequence to achieve high expression.

3.3. Characterization of the promoter of NCgl2319

A 5′ RACE experiment was performed on Pcat-B-EGFP construct to determine transcription initiation site for the promoter. Sequencing results indicated that the transcription initiation site of Pcat-B was located at -91 bp upstream of the start codon ATG. This suggests the -10 region and -35 region of the promoter are CACAGT and ATGTTG upstream of the transcription initiation site (Supplementary Data 1).

In a KEGG database search, we found the metabolic pathways of benzyl alcohol in *C. glutamicum*: from benzyl alcohol, through three intermediates (benzaldehyde, benzoate and (1R, 6S)-1,6-dihydroxycyclohexane-2,4-diene-1-carboxylate) to catechol (Supplementary Fig. 2) [28]. Benzyl alcohol, benzaldehyde, benzoate and catechol (10 mM) were used in the experiments to determine the most efficient inducer to the inducible promoter in Pcat-B. The results show benzyl alcohol had the highest induction efficiency on Pcat-B (Fig. 4A). The induction strength of benzaldehyde and benzoic acid was only 80 % and 60 % that of benzyl alcohol, while Catechol had little induction effect on Pcat-B. We suggest benzyl alcohol, benzaldehyde and benzoate can interact with the regulatory factors of the promoter Pcat-B, but they may have different cell penetration efficiency and the regulatory factors may have a preference for inducers. Overall, benzyl alcohol was shown as the most efficient inducer for Pcat-B.

The promoter regulatory region and the minimal transcriptional initiation region were determined by promoter truncation. We truncated the promoter Pcat-B at its N-terminal side, obtaining a series of truncated promoters with final lengths of 98 bp, 180 bp, 200 bp, 257 bp, 308 bp, 345 bp, 355 bp, 365 bp, 375 bp, 385 bp, 395 bp, 405 bp and 500 bp. The lengths were calculated from N-terminal end of promoters to the beginning of ORF. EGFP expression levels were measured in both induced and non-induced conditions (Fig. 4B). When the length reached to 200 bp, EGFP expression levels are still similar between non-induced and induced conditions, suggesting the region was not covered by transcriptional regulators. In the non-induced condition, EGFP expression increased until the length increased to 257 bp, which could be the minimal functional sequence to initiate transcription (Fig. 4C). When the inducer was added, the expression levels increased until the length reached 345 bp, indicating the 345-bp promoter contains complete regulatory and core promoter regions. The region from – 200 to -345 (A in the ATG start codon was defined as position +1) interacts with

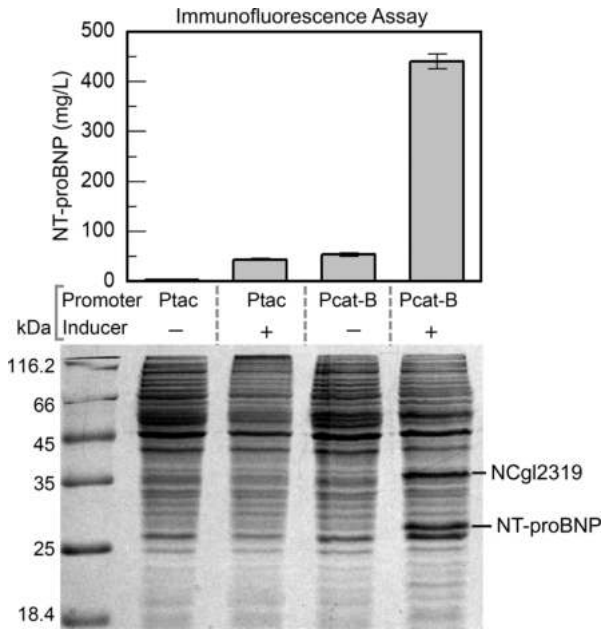

Fig. 6. Expression SUMO-NT-proBNP in Pcat-B and P_{tac} strains. The upper histogram shows the amount of NT-proBNP quantified by Immunofluorescence assays. The lower part is SDS-PAGE analysis of the expression. The target product SUMO-NT-proBNP and by-product catechol 1,2-dioxygenase (encoded by NCgl2319) are indicated.

transcriptional regulators so that when the inducer was added, this region facilitated transcription and greatly increased protein yield, which implies it is an enhancer region interacting with transcriptional activators [29]. This region was also reported to interact with repressors. An AraC-type regulator *RipA* (regulator of iron proteins) was reported to bind to the region around position -342 of NCgl2319 and negatively control the transcription of NCgl2319 [30]. However, the repressor *RipA* responded to iron starvation of the medium. Currently there was no evidence to show the connections between benzyl alcohol and the repressor *RipA*. BenR was another putative transcription activator of NCgl2319 predicted by RegPrecise [31]. It is possible that multiple transcriptional regulators exist in *C. glutamicum* to control the promoter of NCgl2319 and Pcat-B. Further studies will focus on regulation mechanisms of the promoter in Pcat-B and the development of independent inducible Pcat-B expression vectors for application in other bacteria. In addition, strength-tunable regulation might be achieved by changing the abundance of regulators.

3.4. Expression optimization for Pcat-B vector

Different concentrations of benzyl alcohol (1 mM, 10 mM, 15 mM, 20 mM, 25 mM, and 50 mM) were tested to determine the optimum concentration of benzyl alcohol for the induction of Pcat-B (Fig. 5A and 5C). P_{tac} expression vector was used as the standard and positive control. The highest expression level of EGFP in Pcat-B construct was achieved with addition of 10 mM benzyl alcohol. Tunable expression could be achieved by adjusting the concentration of benzyl alcohol, which makes Pcat-B a flexible tool in biological genetic systems. We also observed that when 20 mM or more inducer was added, the expression level significantly decreased. This could be due to the toxic effect of high concentrations of benzyl alcohol on cellular metabolism. Growth curves of those constructs show the cells induced by 10 mM benzyl alcohol had a comparable or even better growing states than IPTG-induced cells (Supplementary Fig. 3).

The expression strength of Pcat-B was compared with P_{tac}, a widely used promoter in *C. glutamicum* (Fig. 5B) [32]. The constructs of Pcat-B-egfp and P_{tac}-egfp were cultured in LBB medium with 10 mM benzyl alcohol or 1 mM IPTG for induction. The expression levels of EGFP in Pcat-B construct was 4.9-times higher than that of P_{tac}, demonstrating Pcat-B was able to express protein in high yield. Benzyl alcohol was used in many studies to investigate membrane fluidity of *E. coli*. In those studies, it was found a wide range of concentrations of benzyl alcohol did not impair cell viability, and did not change the overall protein synthesis in cells up to 30 mM benzyl alcohol [33].

Further studies were conducted by expressing of SUMO-NT-proBNP, a peptide hormone useful for a treatment of decompensated heart failure (Fig. 6) [34]. On the scale of shake-flask culture, Pcat-B constructed strain again showed great advantage in protein production. Enzyme-linked immunofluorescence assays showed that Pcat-B strain produced 440.43 mg/L of NT-proBNP while the yield from P_{tac} strain was 43.61 mg/L, about one-tenth the level of the Pcat-B strain.

The successful production of NT-proBNP in *C. glutamicum* demonstrated that it can be a useful expression system to produce human proteins especially small proteins for pharmaceutical industry. Prokaryotic systems have many remarkable advantages over eukaryotic systems, such as low cost for cultivation, a simple controlling process and the capacity for high-density fermentation [35]. *C. glutamicum* has become a competent host across prokaryotic strains for industrial applications. Various protein secretion systems accompanying with expression system have been developed [36]. As there is no detectable activity of hydrolytic enzymes in the extracellular media, protein products can be well preserved after secretion, greatly simplifying the purification steps. The well-established *C. glutamicum* industrial operation system and facilities suggest that it represents an excellent method for the industrial production of recombinant proteins [37]. The Pcat-B expression system provides high expression in prokaryotic cells for different types of protein commodities.

4. Conclusions

In this study, a benzyl alcohol-inducible promoter was identified from NCgl2319 in *C. glutamicum*. Using a 'context preservation' strategy, we successfully repaired its activity and achieved a high yield in the expression of recombinant proteins. We suggest that this strategy can be applied into other dysfunctional natural promoters in recombinant expression systems. The promoter was fully characterized for tunable utilization. The high strength and compatibility of the promoter was demonstrated by expressing a model protein EGFP and a pharmaceutical protein SUMO-NT-proBNP. The benzyl alcohol-inducible expression system has shown value for the endotoxin free industrial protein production and could work as tunable genetic element in an artificial biological system in *C. glutamicum*.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

Acknowledgement

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.enzmictec.2020.109651.

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