Development of a Hyperosmotic Stress Inducible Gene Expression System by Engineering the MtrA/MtrB-Dependent NCgl1418 Promoter in Corynebacterium glutamicum

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Corynebacterium glutamicum is an important workhorse for industrial production of diversiform bioproducts. Precise regulation of gene expression is crucial for metabolic balance and enhancing production of target molecules. Auto-inducible promoters, which can be activated without expensive inducers, are ideal regulatory tools for industrial-scale application. However, few auto-inducible promoters have been identified and applied in C. glutamicum. Here, a hyperosmotic stress inducible gene expression system was developed and used for metabolic engineering of C. glutamicum. The promoter of NCgl1418 (PNCgl1418) that was activated by the two-component signal transduction system MtrA/MtrB was found to exhibit a high inducibility under hyperosmotic stress conditions. A synthetic promoter library was then constructed by randomizing the flanking and space regions of PNCgl1418, and mutant promoters exhibiting high strength were isolated via fluorescence activated cell sorting (FACS)-based high-throughput screening. The hyperosmotic stress inducible gene expression system was applied to regulate the expression of lysE encoding a lysine exporter and repress four genes involved in lysine biosynthesis (gltA, pck, pgi, and hom) by CRISPR interference, which increased the lysine titer by 64.7% (from 17.0 to 28.0 g/L) in bioreactors. The hyperosmotic stress inducible gene expression system developed here is a simple and effective tool for gene auto-regulation in C. glutamicum and holds promise for metabolic engineering of C. glutamicum to produce valuable chemicals and fuels.

Keywords: Corynebacterium glutamicum, hyperosmotic stress, inducible promoter, MtrA, fluorescence activated cell sorting, lysine

INTRODUCTION

The nonpathogenic Gram-positive Corynebacterium glutamicum is a biosafe strain recognized by FDA and widely used in biomanufacturing of amino acids, organic acids, proteins, and other chemicals (Woo and Park, 2014; Lee et al., 2016; Tsuge and Matsuzawa, 2021). Regulation and optimization of target gene expression is crucial for balance of metabolic pathway and...
improvement of product biosynthesis. Promoters with different properties are one of the most effective tools to control gene expression. At present, a series of promoters have been identified or developed to regulate gene expression in *C. glutamicum* (Becker et al., 2005; Kind et al., 2010; Patek and Nesvera, 2011; Dostalova et al., 2017; Zhang et al., 2018; Sun et al., 2020; Wang et al., 2020; Wiechert et al., 2020a, b; Henke et al., 2021). In general, there are two types of promoters: constitutive promoters and inducible promoters. The strong constitutive promoters, such as the *tuf* and *sod* promoters, are widely used to enhance the expression of target genes (Becker et al., 2005; Kind et al., 2010). However, in some cases, gene expression controlled by strong constitutive promoters may cause cellular burden and metabolic unbalance, which hinders bioproduction (Giacalone et al., 2006; Terpe, 2006). Compared to constitutive promoters, inducible promoters can initiate the gene expression at any time when required, so they are preferred for the regulation and redistribution of metabolic flux and the expression of proteins with cytotoxicity (Lipscomb et al., 2004). At the moment, *lac*, *tre*, and other inducible promoters are extensively applied in the metabolic regulation of *C. glutamicum* (Ben Samoun et al., 1999; Okibe et al., 2010; Lausberg et al., 2012; Sun et al., 2020; Wiechert et al., 2020b; Henke et al., 2021), but there are also limitations. The requirement of inducers that are toxic (such as isopropyl-β-D-thiogalactopyranoside (IPTG)) or exhaustible (such as glucose) limits the use in large scale. Besides, inducers are usually added during the cultivation, so monitoring the cell growth state and optimizing the timing of inducer addition are required (Nocadello and Swennen, 2012). Therefore, there is a demand in development of auto-inducible systems without the use of external inducers.

Recently, auto-inducible promoters that can automatically turn on gene expression in response to environmental factors, metabolites, and cell growth state have been identified and applied in several bacteria. For example, a dynamic turn-off switch (dTFS) and a dynamic turn-on switch (dTNS) in *Escherichia coli* were constructed using the growth phase-dependent promoter and degron (Lemke et al., 2011). This bifunctional molecular switch was used to uncouple cell growth from the biosynthesis of shikimic acid and glucaric acid (Hou et al., 2020). Pyruvate-responsive genetic circuits were constructed in *Bacillus subtilis* by the hybrid promoter that contained the PdhR-binding site and the pyruvate-responsive transcription factor PdhR from *E. coli*, which were used to balance and optimize the metabolic flux toward the production of glucaric acid (Ogasavara et al., 2007; Xu et al., 2020a). An endogenous quorum-sensing (QS) based CRISPRi circuit in *Streptomyces* was constructed, in which the *dacs*9 gene was regulated by a native QS signal-responsive promoter. The system was used for downregulating three key nodes in essential pathways to divert metabolic flux toward rapamycin biosynthesis (Tian et al., 2020). In *C. glutamicum*, some auto-inducible promoters have also been reported and applied. For example, based on the LysR-type transcriptional regulator (LTTR) LysG and the *lysE* promoter with the LysG-binding site, a sensor suitable for intracellular lysine detection was developed (Binder et al., 2012). Two growth-regulated promoters (*P*<sub>CP_2836</sub> and *P*<sub>CP_2854</sub>) had been identified (Kim et al., 2016; Ma et al., 2018). The *P*<sub>*gltH*<sup>3141</sup> promoter was verified with the production of glutathione S-transferase as a model protein, and the *P*<sub>*gltH*<sup>3146</sup> promoter was used for improving the production of valine and S-aminolevulinic acid (Kim et al., 2016; Ma et al., 2018; Zhang et al., 2020).

With strict control of fermentation conditions such as pH and dissolved oxygen, the titer of target metabolites in the bioreactors can reach 100 g/L or even 200 g/L. Lys production by *C. glutamicum*, for example (Xu et al., 2020b). Therefore, in the mid- and late-stage of fermentation, the accumulation of high concentrations of products or the feeding of substrates will inevitably lead to a high-salt or hyperosmotic stress, which can be used as a natural inducer that exits in almost all fermentation processes (Varela et al., 2003; Chung et al., 2017). However, hyperosmotic stress inducible promoters have not been identified so far. MrA/MrB, one of the two-component systems that are highly conserved in corynebacteria and mycobacteria, regulates the expression of genes involved in osmoprotection (Moker et al., 2004; Bott and Brocker, 2012). Some target genes and the consensus binding sites of MrA have also been identified in *C. glutamicum* (Brocker et al., 2011). However, the MrA/MrB-dependent promoters responding to the hyperosmotic stress have not been identified and applied in gene regulation.

In this study, a hyperosmotic stress inducible gene expression system based on the hyperosmotic stress inducible promoter was developed in *C. glutamicum*. Among eight promoters regulated by the two-component system MrA/MrB, the promoter of NCgl1418 (*P*<sub>NCgl1418</sub>) showed high expression intensity and induction activity under a hyperosmotic stress. The core sequence of *P*<sub>NCgl1418</sub> was identified and the activity and inducibility was further enhanced by mutagenesis and fluorescence activated cell sorting (FACS)-based high-throughput screening. To demonstrate an application of this inducible system in metabolic engineering of *C. glutamicum*, it was used for overexpression of lysine exporter and CRISPR-dCpf1-mediated multiplex gene repression of four endogenous genes (Li et al., 2020) to maximize lysine production. This MrA/MrB-dependent hyperosmotic stress inducible system may also be useful for gene regulation in microorganisms beyond *C. glutamicum*.

**MATERIALS AND METHODS**

**Bacterial Strains and Cultivation Conditions**

The bacterial strains used in this study are listed in **Supplementary Table S1**. *E. coli* Trans1-T1 and Trans DB 3.1 were used as the host for cloning and plasmid maintenance. Wild-type *C. glutamicum* ATCC 13032 was used as the host for gene expression and promoter screening. *C. glutamicum* SCgL30, the derivative of *C. glutamicum* ATCC 13032 with a feedback deregulated aspartokinase (T311; Becker and Wittmann, 2012), was used for lysine production. *E. coli* was cultivated at 37°C in Luria-Bertani (LB) medium (5 g/L yeast extract, 10 g/L tryptone, and 10 g/L NaCl). Kanamycin (50 μg/ml) or chloramphenicol (20 μg/ml) was added to LB.
medium as required. *C. glutamicum* was cultivated at 30°C in BHI medium containing 37 g/L bovine brain heart extract, 10 g/L (NH₄)₂SO₄, 0.2 g/L K₂HPO₄, 0.3 g/L NaH₂PO₄, and 0.5 g/L MgSO₄·7H₂O. For fluorescence intensity determination, *C. glutamicum* was cultivated in a defined medium (CGXII medium) containing 50 g/L glucose, 2 g/L yeast extract, 16.5 g/L NH₄Cl, 5 g/L urea, 1 g/L KH₂PO₄, 1 g/L K₂HPO₄, 42 g/L MOPS, 0.25 g/L MgSO₄, 0.01 g/L FeSO₄·7H₂O, 0.01 g/L MnSO₄·H₂O, 0.001 g/L ZnSO₄·7H₂O, 0.2 mg/L CuSO₄·0.02 mg/L NiCl₂·6H₂O, 0.01 g/L CaCl₂, 0.03 g/L proteocatechue acid, 0.2 mg/L biotin, and 0.1 mg/L vitamin B1. Kanamycin (25 μg/mL), chloramphenicol (5 μg/mL) or IPTG (1 mM) was added when necessary.

**Plasmid Manipulation**

The plasmids and primers used in this study are listed in [Supplementary Tables S1 and S2](#), respectively. The promoter regions [about 300 bp upstream of the coding sequence (CDS)] of *abgT*, *csbD*, *betP*, *NCgl1418*, *NCgl1756*, *NCgl1838*, *NCgl2841*, and *proP* were amplified from the genomic DNA of *C. glutamicum* ATCC 13032 by PCR with eight primer pairs. The backbone of pEC-02 was amplified with the primer pair tac-F/R, and then self-cyclized to generate the plasmid pXM-02. Similarly, array-F1/R1, array-F2/R2, and array-F4/R4 were annealed, and assembled into BbsI-digested pXM-02 backbone using a Golden Gate assembly method, resulting in the plasmid pXM-02-RFP. All PCR fragments were purified and ligated to generate the plasmid pXM-02-RFP. Two oligonucleotides (RFP-F/R) were annealed and assembled into BbsI-digested pXM-02-RFP backbone using a Golden Gate assembly method, resulting in the plasmid pXM-02-RFP. All PCR fragments were purified and ligated to generate the plasmid pXM-02-RFP.

The two plasmids (pgRNA-mtrA and pCas9) were transformed into *E. coli* BL21 (DE3) to generate the plasmid pXM-PgRNA-mtrA. The backbone of pXM-PgRNA-mtrA was amplified with the primer pair pgRNA-mtrA-del-up-F/R and then self-cyclized to generate the plasmid pXM-PgRNA-mtrA. The pEC-02 backbone and the pXM-PgRNA-mtrA plasmid were amplified with the primer pair tac-F/R, and then self-cyclized to generate the plasmid pXM-PgRNA-mtrA. The backbone of pEC-02 was amplified with the primer pair tac-F/R, and then self-cyclized to generate the plasmid pXM-PgRNA-mtrA. The backbone of pEC-02 was amplified with the primer pair tac-F/R, and then self-cyclized to generate the plasmid pXM-PgRNA-mtrA.

A previously developed CRISPR-dCpf1 system (Li et al., 2020) was modified to repress the expression of target genes in response to the hyperosmotic stress. To construct an all-in-one CRISPRi tool plasmid, the backbone of pXM-07 expressing dCpf1 was divided into three parts, which were amplified with the primer pairs pXM-07-F1/R1, pXM-07-F2/R2, and pXM-07-F3/R3, respectively. A gRNA cassette consisting of a constitutive promoter (P_{ifh}), two direct repeats (DRs), a *cdB* flanked by two BbsI sites, and a terminator was amplified from pEC-02 (Li et al., 2020) with the primer pair ccdB-F/R. These four PCR fragments were purified and ligated to generate the plasmid pXM-dCpf1. Two oligonucleotides (RFP-F/R) were annealed and assembled into BbsI-digested pXM-dCpf1 backbone using a Golden Gate assembly method, resulting in the plasmid pXM-dCpf1-RFP.

The two plasmids (pgRNA-mtrA and pCas9) were transformed into *E. coli* BL21 (DE3) to generate the plasmid pXM-PgRNA-mtrA. The backbone of pXM-PgRNA-mtrA was amplified with the primer pair pgRNA-mtrA-del-up-F/R and then self-cyclized to generate the plasmid pXM-PgRNA-mtrA. The backbone of pEC-02 was amplified with the primer pair tac-F/R, and then self-cyclized to generate the plasmid pXM-PgRNA-mtrA. The backbone of pEC-02 was amplified with the primer pair tac-F/R, and then self-cyclized to generate the plasmid pXM-PgRNA-mtrA. The backbone of pEC-02 was amplified with the primer pair tac-F/R, and then self-cyclized to generate the plasmid pXM-PgRNA-mtrA.
cured from the edited mutant before it was used for subsequent experiments.

**Construction of Synthetic Promoter Libraries**

The synthetic promoter libraries of P\textsubscript{NCgl4418} were constructed using the primer pairs P-Library-F1/R1 and P-Library-F2/R2 containing degenerated oligonucleotides (Ns) by PCR using pX-M-P\textsubscript{NCgl4418-gfp} as a template. The PCR product was self-ligated with T4 ligase. The ligation products were transformed into strain E. coli Trans1-T1. The quality of randomization was checked by sequencing 24 randomly picked transformants. The synthetic promoter libraries were extracted and then transformed into C. glutamicum ATCC 13032 by electroporation.

**FACS Screening**

The C. glutamicum library was inoculated into BHI medium and cultivated overnight at 30°C and 220 rpm. The overnight cultures were transferred into 24-well plates with 1 ml CGXIIY medium containing 0.6 M Na\textsubscript{2}SO\textsubscript{4} to an optical density at 600 nm (OD\textsubscript{600}) of 0.5. Then, cells were cultivated for 6 h at 30°C and with shaking at 800 rpm in INFORS Microtron (INFORS HT Multitron Pro, Switzerland). The cell suspension was diluted by proper times with distilled water and sorted on FACS (MoFlo XDP, Beckman Coulter, Inc., Miami, FL, United States) using green fluorescent protein (GFP) as the reporter protein. The cells were excited by 488-nm laser and detected through a 520 band-pass filter. In the first-round screening, cells showing high fluorescence intensity (the top 0.01%) were sorted (positive sorting). The sorted cells were poured directly into 5 ml BHI medium and cultivated overnight, and then the cells were transferred into 24-well plates with 1 ml CGXIIY medium for the next round of screening. In the second-round screening, cells showing low fluorescence intensity (the bottom 1%) were sorted (negative sorting). Similarly, the third-round screening (positive sorting) was performed. After the three-round sorting, the sorted cells were spread on BHI agar plate and incubated at 30°C.

**Fluorescence Intensity Determination**

The C. glutamicum was inoculated into BHI medium and cultivated overnight at 30°C and 220 rpm. The overnight cultures were transferred into 24-well plates with 1 ml CGXIIY medium with or without 0.6 M Na\textsubscript{2}SO\textsubscript{4} to an OD\textsubscript{600} of 0.5. After cultivated at 30°C and with shaking at 800 rpm for 18 h, cells were harvested and diluted by proper times with distilled water. GFP fluorescence intensities were determined using a microplate reader (SpectraMax M5, Molecular Devices, λ excitation = 488 nm, λ emission = 520 nm). The fluorescence intensities were normalized with OD\textsubscript{600}.

**Lysine Production in 24-Well Plates**

C. glutamicum SCgL30 with a feedback deregulated aspartokinase (T311I; Becker and Wittmann, 2012) was used for lysine production. Strain SCgL30 and its derivatives were cultivated in BHI medium at 30°C and with shaking at 220 rpm. The overnight cultures were transferred into 24-well plates containing 1 ml fermentation medium each well to an initial OD\textsubscript{600} of 0.5. The fermentation medium contains 80 g/L glucose, 8 g/L yeast extract, 9 g/L urea, 1.5 g/L K\textsubscript{2}HPO\textsubscript{4}, 0.01 g/L MnSO\textsubscript{4}, 0.6 g/L MgSO\textsubscript{4}·7H\textsubscript{2}O, 0.01 g/L FeSO\textsubscript{4}·7H\textsubscript{2}O, and 42 g/L MOPS (Li et al., 2020). Then, cells were cultivated for 24 or 36 h at 30°C and with shaking at 800 rpm in INFORS Microtron (INFORS HT Multitron Pro, Switzerland).

**Lysine Production in 1-L Bioreactors**

Colonies grown on a BHI plate at 30°C for 24 h were picked and used to inoculate 50 ml LSS1 medium (Ohnishi et al., 2002) in 500 ml shake flasks. The cultures were then cultivated overnight at 30°C and with shaking at 220 rpm.! The cultures were transferred into a 1-L jar bioreactor [T&8]-MiniBox, T&8 Bio-engineering (Shanghai) Co., Ltd., China) containing 500 ml LPG1 medium (Ohnishi et al., 2002) with an inoculation volume of 10%. After the sugar initially added to the bioreactor was exhausted, glucose solution (50%, w/v) containing 0.5 mg/L biotin was continuously fed to maintain the glucose concentration at about 1.5% (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} solution (5.56%, w/v) also was also fed to supply nutrient source for lysine biosynthesis and cell growth. The fermentation was performed at 34°C with an agitation speed of 900 rpm and an aeration at 2.5 L/min. The pH was maintained at 7.3 with NH\textsubscript{4}OH. LSS1 medium (Ohnishi et al., 2002) contains 50 g/L sucrose, 40 g/L corn steep liquor, 8.3 g/L (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 1 g/L urea, 2 g/L KH\textsubscript{2}PO\textsubscript{4}, 0.83 g/L MgSO\textsubscript{4}·7H\textsubscript{2}O, 10 mg/L FeSO\textsubscript{4}·7H\textsubscript{2}O, 1 mg/L CuSO\textsubscript{4}·5H\textsubscript{2}O, 10 mg/L ZnSO\textsubscript{4}·7H\textsubscript{2}O, 10 mg/L β-alanine, 5 mg/L vitamin B3, 1.5 mg/L vitamin B1, 0.5 mg/L biotin, and 30 g/L CaCO\textsubscript{3}. LPG1 medium (Ohnishi et al., 2002) contains 50 g/L glucose, 20 g/L corn steep liquor, 15 g/L (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 1 g/L urea, 2.5 g/L KH\textsubscript{2}PO\textsubscript{4}, 0.75 g/L MgSO\textsubscript{4}·7H\textsubscript{2}O, 50 mg/L FeSO\textsubscript{4}·7H\textsubscript{2}O, 13 mg/L MnSO\textsubscript{4}·5H\textsubscript{2}O, 50 mg/L CaCl\textsubscript{2}·2H\textsubscript{2}O, 6.3 mg/L CuSO\textsubscript{4}·5H\textsubscript{2}O, 1.3 mg of ZnSO\textsubscript{4}·7H\textsubscript{2}O, 5 mg/L NiCl\textsubscript{2}·6H\textsubscript{2}O, 1.3 mg/L CoCl\textsubscript{2}·6H\textsubscript{2}O, 1.3 mg of (NH\textsubscript{4})\textsubscript{2}Mo\textsubscript{7}O\textsubscript{24}·4H\textsubscript{2}O, 23 mg/L β-alanine, 14 mg/L vitamin B3, 7 mg/L vitamin B1, and 0.5 mg/L biotin. Samples were taken periodically and glucose and lysine concentrations were quantified using an SBA-40D biosensor analyzer (Institute of Biology of Shandong Province Academy of Sciences, Shandong, China). Cell biomass was determined as OD\textsubscript{600} with a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan) after proper dilution with distilled water.

**RESULTS**

**Identification of Hyperosmotic Stress Inducible Promoters**

To identify the hyperosmotic stress inducible promoters, we tested the promoters of the genes showing MtrA-dependent behaviors. Eight promoters (P\textsubscript{abgT}, P\textsubscript{csbD}, P\textsubscript{betP}, P\textsubscript{NCgl4418}, P\textsubscript{NCgl4720}, P\textsubscript{NCgl4418}, P\textsubscript{NCgl2441}, and P\textsubscript{mtr}), which control the expression of abgT (secondary transporter of the AbgT family), csbD, betP
Huang et al. Hyperosmotic Stress-Inducible Promoters for Corynebacterium

Frontiers in Microbiology | www.frontiersin.org 5 July 2021 | Volume 12 | Article 718511

(glycine betaine transporter), NCgl1418, NCgl1756, NCgl1838, NCgl2841, and proP (proline/ectoine carrier), respectively, were selected based on a previous study (Brocker et al., 2011). These genes showed significantly decreased transcription levels (<10% of the control) upon the deletion of mtrA in C. glutamicum. Besides, the MtrA binding sites of these promoters have also been identified (Brocker et al., 2011). Since most of the exact promoters and regulatory regions of those genes have not been fully defined yet, the ~300 bp upstream of each CDS was investigated. In order to assess these MtrA-dependent promoters, GFP was used as a reporter to study the transcriptional strength of these promoters with or without 0.6 M NaCl. P_abgT, P_NCgl1418, P_NCgl1838, and P_proP showed more than 2.0-fold NaCl-induced expression activity. Among them, P_NCgl1418 showed the highest induced expression intensity (Figure 1). Therefore, we chose P_NCgl1418 for further analysis.

Characterization of the NCgl1418 Promoter

Next, the effects of different types or concentrations of inducers on induction of P_NCgl1418 were investigated. Using GFP as a reporter, it was found that sulfate was the best inducer, which induced the expression activity of P_NCgl1418 up to 4.0-fold (Figure 2A). P_NCgl1418 showed obvious gradient induction activity in the range of 0~0.6 M Na2SO4 (Figure 2B). Compared with the endogenous strong constitutive promoter P_tuf and the widely used IPTG-inducible promoter P_nar (Becker et al., 2005; Kind et al., 2010; Rytter et al., 2014), P_NCgl1418 showed about 62% expression strength upon hyperosmotic stress induction (Figure 2C). These results demonstrate that P_NCgl1418 is a relatively strong hyperosmotic stress inducible promoter, which has application potential for initiating the expression of target genes during the fermentation process.

To determine the key functional region of P_NCgl1418, three truncated variants (94, 145, and 203 bp) were constructed and used for GFP expression (Figure 3A). Although the 94 bp P_NCgl1418 promoter contained the core −35 and −10 regions (Pfeifer Sancar et al., 2013), the function of the promoter completely lost. The 145 bp promoter contained the MtrA binding site but showed a decreased expression activity (74%) under hyperosmotic stress. In contrast, the 203 bp promoter maintained almost the same activity (94%) to the whole-length promoter (243 bp) under hyperosmotic stress (Figure 3B). To confirm the necessity of MtrA for P_NCgl1418 functioning, a mtrA-deleted C. glutamicum strain was constructed and the plasmid harboring the P_NCgl1418-controlled gfp cassette was transformed into the mutant. As expect, no GFP expression could be detected with mtrA deletion (Supplementary Figure S1). These results suggest that MtrA and its specific binding site are essential to the inducible activity of P_NCgl1418. However, considering the partial activity loss of the 145 bp truncation, unknown mechanisms may also have an impact on the promoter.

Construction of P_NCgl1418 Libraries and Isolation of Variants With Enhanced Inducible Activity

Although P_NCgl1418 exhibited a relatively high inducible activity, its expression intensity was still lower than that of P_nar and P_nuc, which may hinder the application in high-level gene expression. Therefore, further engineering of this promoter is needed to increase its strength. To engineer the P_NCgl1418, random promoter libraries were constructed based on the

![FIGURE 1 | Screening of hyperosmotic stress inducible promoters. Expression of gfp was controlled by different MtrA/MtrB-dependent promoters (P_abgT, P_csbD, P_betP, P_NCgl1418, P_NCgl1756, P_NCgl1838, P_NCgl2841, and P_proP) in C. glutamicum with or without high salinity stress induction. Cell fluorescence intensity was normalized to cell density and the background value of the strain containing the control vector pXM-con-gfp without a promoter was deducted. All data represent mean values from three biological replicates including standard deviations (SDs).](image-url)
243 bp wild-type promoter. The deduced −10 and −35 regions of P_NCgl1418 were fixed to allow sigma factor binding, and the 29 bp spacer and left flanking regions were designed to have fully randomized nucleotides (Figure 4A). The randomized promoters were cloned to the upstream of the GFP reporter gene and subjected to a three-step positive-negative-positive screening to identify promoter variants with improved induction activity and expression strength. During this screening process, positive screening in the presence of 0.6 M Na₂SO₄ was conducted to identify high-fluorescence variants (isolation of the top 0.01% fluorescing cells) and the negative screening in the absence of 0.6 M Na₂SO₄ was conducted to exclude the variants with too high fluorescence intensity without induction (isolation of the bottom 1% non-fluorescing cells; Figure 4B). After three rounds of screening, the sorted cells were cultivated on agar plates, and 90 clones were randomly picked and characterized. After a secondary screening in microtiter plates, a total of 10 promoter variants with different sequences (Supplementary Figure S2) and inducible activities were obtained (Figure 4C). One promoter variant A10
Huang et al. Hyperosmotic Stress-Inducible Promoters for Corynebacterium

Frontiers in Microbiology | www.frontiersin.org
7 July 2021 | Volume 12 | Article 718511

\[ \text{P}_{\text{NCgl1418-A10}} \] showed the highest inducible activity and was chosen for further analysis. \( \text{P}_{\text{NCgl1418-A10}} \) was then compared with the strong constitutive promoter \( \text{P}_{\text{tuf}} \) and IPTG-inducible promoter \( \text{P}_{\text{tac}} \) with or without 0.6 M \( \text{Na}_{2}\text{SO}_{4} \). The expression of GFP under the control of \( \text{P}_{\text{NCgl1418-A10}} \) showed an 8.5-fold induction by 0.6 M \( \text{Na}_{2}\text{SO}_{4} \). The induced GFP expression level was approximately 1.8-fold higher than those of \( \text{P}_{\text{tuf}} \) and \( \text{P}_{\text{tac}} \) (Figure 4C).

**Application of the Hyperosmotic Stress Inducible Promoter to Optimize Lysine Biosynthesis**

To demonstrate the usability of the hyperosmotic stress inducible promoters in metabolic engineering, the wild-type \( \text{P}_{\text{NCgl1418}} \) and \( \text{P}_{\text{NCgl1418-A10}} \) variant were used to regulate five genes related to lysine biosynthesis and excretion in \( C. \text{glutamicum} \) (Figure 5A). One target gene \( \text{lysE} \) encodes a lysine efflux protein and overexpression of \( \text{lysE} \) can increase lysine production by enhancing excretion of lysine (Zhou and Zeng, 2015). The other four genes, \( \text{gltA, pck, pgi, and hom} \), are involved in the metabolic flux competition with lysine biosynthesis and their repression by CRISPRi has been proven to enhance the lysine production (Li et al., 2020).

Firstly, we constructed an all-in-one CRISPRi tool plasmid \( \text{pXM-}d\text{Cpf1} \) based on the previously developed two plasmids system expressing \( d\text{Cpf1} \) under the control of IPTG-inducible \( \text{P}_{\text{lac}} \) and crRNA array under the control of constitutive \( \text{P}_{11F} \) (Li et al., 2020). To determine the gene repression efficiency of the all-in-one CRISPRi system, a crRNA targeting the template strand of red fluorescent protein (RFP) encoding gene was designed and assembled to \( \text{pXM-}d\text{Cpf1} \). A repression efficiency of 77% was achieved (Supplementary Figure S3), which was similar with the previous system using two plasmids. For combinational repression of the four target genes (gltA, pck, pgi, and hom), a crRNA array harboring four spacers was assembled to \( \text{pXM-}d\text{Cpf1} \). To achieve hyperosmotic stress inducible gene repression, the IPTG-inducible \( \text{P}_{\text{lac}} \) was replaced with \( \text{P}_{\text{NCgl1418}} \) and \( \text{P}_{\text{NCgl1418-A10}} \), respectively, for controlling \( d\text{Cpf1} \) expression. The two CRISPRi plasmids and a control plasmid without targeting crRNA array were individually transformed into the lysine-producing strain SCgL30 with a feedback deregulated aspartokinase (T311I; Becker and Wittmann, 2012). Fermentation in 24-well plates was performed to evaluate lysine.

**FIGURE 4** | FACS-based positive/negative screening of the random promoter library to identify \( \text{P}_{\text{NCgl1418}} \) promoter variants with enhanced inducible activity.

(A) Design of the random promoter library. (B) FACS plots of the three rounds of positive/negative screening. (C) Characterization of the isolated promoter variants. Cell fluorescence intensity was normalized to cell density and the background value of the strain containing the control vector \( \text{pXM-con-gfp} \) without a promoter was deducted. All data represent mean values from three biological replicates including SDs.
production. Without addition of 0.6 M Na₂SO₄ to provide a hyperosmotic stress, repression of four target genes using P_NCgl1418 enhanced lysine titer and yield by 37 and 45%, respectively (Figure 5B). When the hyperosmotic stress was exerted to cells by adding 0.6 M Na₂SO₄, repression of four target genes enhanced lysine titer and yield by 79 and 62%, respectively (Figure 5B). The lysine titer was improved by 92% (reached 4.5 g/L) when the stronger promoter variant P_NCgl1418-A10 was used in the presence of hyperosmotic stress. The results suggest that the hyperosmotic stress inducible promoters can be used for efficiently regulating gene expression under high salt conditions for metabolic engineering purposes.

For further testing the application of the hyperosmotic stress inducible promoters in gene upregulation, lysE was selected and overexpressed in SCgL30. Without the addition of 0.6 M Na₂SO₄, overexpression of lysE slightly increased the lysine titer and yield. However, under the hyperosmotic stress, lysE overexpression controlled by P_NCgl1418-A10 exhibited much higher lysine titer (2.3-fold) and yield (about 2.0-fold) compared with the control strain (Figure 5B). Finally, overexpression of lysE and repression of gltA, pck, pgI, and hom by CRISPRi were simultaneously performed for lysine production using the strain SLDEA10. Under the hyperosmotic stress, this combinational strategy improved lysine titer and the yield by 2.5- and 4.8-fold compared to the control, respectively (Figure 5B). It was noticed that the lysine titers of strain SLDEA10 cultivated with or without 0.6 M Na₂SO₄ were almost the same. This is may be due to the slower growth rate of strain SLDEA10 under hyperosmotic stress (Supplementary Figure S4). The growth retardation might be caused by the higher expression of dCpf1 and LysE or the stronger repression of the endogenous genes under hyperosmotic stress. However, the lysine yield reached 388.7 mM/L, which improved by 4.8-fold when 0.6 M Na₂SO₄ was added (Figure 5B). The results indicate that the promoter variant P_NCgl1418-A10 has strong inductivity and the rebalance between growth and production is necessary to increase the performance of engineered strains.

**Feed Batch Fermentation of the Lysine Producer With the Auto-Inducible System**

Because of the limited substrate supplement and the lack of fermentation condition control, fermentation in 24-well plates cannot result in high-titer lysine production. Therefore, 0.6 M Na₂SO₄ was added to provide a hyperosmotic stress to induce gene expression controlled by P_NCgl1418 and P_NCgl1418-A10. Fed-batch fermentations are usually used to obtain high concentrations of products, which can serve as inducers for hyperosmotic stress inducible promoters such as P_NCgl1418-A10. To demonstrate the auto-inducible system based on P_NCgl1418-A10, fed-batch
fermentations without exogenous Na$_2$SO$_4$ addition were conducted in 1-L bioreactors using the best lysine producer strain SLDEA10 and a control strain SLDEcon2 without the auto-inducible CRISPRi and lysE overexpression system. Overall, SLDEA10 had a better balance between growth and production than the control strain (Figure 6). Strain SLDEcon2 entered the stationary phase at 15 h with a relatively high OD$_{600nm}$ peak value, while the highest OD$_{600nm}$ was significantly reduced by 32.3% for strain SLDEA10 (Figure 6). On the contrary, the lysine biosynthesis rate of SLDEA10 was significantly higher than that of strain SLDEcon2 in the mid- and late-stage. After 33 h fermentation, 28.0 g/L of lysine was produced by strain SLDEA10, representing a 64.7% improvement compared to strain SLDEcon2 (17.0 g/L; Figure 6). These results suggest that the metabolic flux is directed from biomass synthesis to product synthesis by using the auto-inducible system based on the hyperosmotic stress inducible promoter.

DISCUSSION

In the last few decades, C. glutamicum has been engineered for the production of a variety of value-added products, including amino acids, organic acids, polymer precursors, and proteins (Baritugo et al., 2018; Lee and Kim, 2018). Gene expression regulation technologies based on CRISPR and synthetic small RNAs for C. glutamicum have been developed (Liu et al., 2017; Wang et al., 2018; Gauttam et al., 2019; Sun et al., 2019; Li et al., 2020). By combining these technologies with metabolic- or QS-responding elements, dynamic regulation strategies have been developed for precise regulation of gene expression to maximize the biosynthesis of target products (Gupta et al., 2017; Chen et al., 2018; Shen et al., 2019; Liang et al., 2020; Tian et al., 2020; Hartline et al., 2021). However, the metabolic-responding elements are specific for certain metabolites and thus they are not universal for various fermentation processes. Although QS systems have good universality, their application in large-scale fermentation usually suffers from the low threshold. As an alternative, the environmental parameter-responding elements have the advantages of high universality and good controllability, which can be used to develop universal dynamic regulation systems. At present, dynamic regulation strategies based on external environmental conditions such as temperature, dissolved oxygen, pH, and illumination have been successfully developed and applied in metabolic engineering (Jong Uk et al., 2008; Binder et al., 2016; Hwang et al., 2017; Harder et al., 2018; Zhao et al., 2018; Banares et al., 2020). However, in large-scale fermentation, these environmental factors including temperature, dissolved oxygen, and pH mostly need to be controlled at a steady level to maintain cellular activity, and optogenetics tools are limited by the nonhomogeneity in large fermenters. Hyperosmotic stress, which commonly presents in the mid- and late-phase of almost all the fermentation processes, can serve as a naturally occurring inducer. Compared with the promoters based on metabolic- or QS-responding elements and those responding to external environmental conditions such as temperature, dissolved oxygen, pH, and illumination, the hyperosmotic stress inducible promoters have good universality and economy in the large-scale industrialization applications. In this study, a hyperosmotic stress inducible gene expression system was developed in C. glutamicum. The promoter of NCgl1418 identified here exhibits high expression intensity and induction activity under hyperosmotic stress caused by different salt inducers. More importantly, the induction activity was closely related to product concentration, which can be conveniently used to regulate the expression of key genes according to the product concentration. Considering the different expression intensities and induction activities by different types or concentrations of salts, optimization tests for different media may be required in practical applications.

Promoters are the core elements of the dynamic regulation systems and synthetic biology researches. Identification and modification of novel promoters with special functions can provide necessary tools for the development of new gene circuits and hyperproducing strains. At present, omics analysis
under different culture conditions and bioinformatics analysis of specific recognition sequences of transcriptional regulators are the main strategies for the identification of new promoters (Dahl et al., 2013; Liu et al., 2015; Kim et al., 2016; Ma et al., 2018). However, the strength and induction activity of natural promoters are often not sufficient for practical applications. To engineer more suitable promoters, random mutation followed by high-throughput screening and redesign of the natural promoters with components from other different promoters are usually performed (Kim et al., 2016; Zhang et al., 2018; Henke et al., 2021). In this study, for isolation of engineered promoters with enhanced inducibility by hyperosmotic stress, we used a FACS screening strategy that combined three rounds of positive and negative screening. The isolated promoters showed up to a 3.4-fold increased GFP expression level compared to the original NCgl1418 promoter with much higher inducibility (up to 8.5-fold). As a proof-of-concept, lysine production was improved by regulating key genes expression with the hyperosmotic stress inducible promoters, demonstrating the great potential in metabolic engineering of C. glutamicum. Also, we observed leaky expression of the NCgl1418 promoter, like all the other kinds of inducible promoters. For a stricter control of the NCgl1418 promoter, conditional overexpression of its regulator MtrA/MtrB may be a useful strategy. In addition, promoter variants with low-level leaky expression could also be further screened by FACS. In conclusion, we identified the MtrA/MtrB-dependent NCgl1418 promoter as a hyperosmotic stress inducible promoter and developed a hyperosmotic stress inducible gene expression system in C. glutamicum. To the best of our knowledge, this is the first report on the development of a hyperosmotic stress inducible promoter in C. glutamicum. We believe this hyperosmotic stress inducible gene expression system will contribute to extend the usefulness of C. glutamicum in the industrial-scale production of various value-added biochemicals.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

JH, JC, YW, PZ, and JS conceptualized the project, designed the study, and composed the manuscript. JH, JC, TS, YZ, and NC conducted the experiments and collected data. JH, JC, YW, XN, WP, and JL performed the data analysis. PZ, JS, and SH provided the critical feedback on the manuscript and resources. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.718511/full/supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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