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Downregulation of T7 RNA polymerase transcription enhances pET-based recombinant protein production via suppression of Escherichia coli BL21 (DE3) programmed cell death

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Abstract

E. coli BL21 (DE3) is an excellent and widely used host for recombinant protein production. Many variant hosts were developed from from BL21 (DE3), but improving the expression of specific proteins remains a major challenge in biotechnology. In this study, we found that when BL21 (DE3) overexpressed glucose dehydrogenase (GDH), a significant industrial enzyme, serious autolysis was induced. Subsequently, we observed this phenomenon in the expression of 10 other recombinant proteins. This precludes a further increase of the produced enzyme activity by extending the fermentation time, which is not conducive to the reduction of industrial enzyme production costs. The membrane structure and mRNA expression analysis showed that cells suffered programmed cell death (PCD) during autolysis period. However, blocking three known PCD pathway in BL21 (DE3) cannot alleviate autolysis completely. Furthermore, we attempted to develop a strong expression host resistant to autolysis by controlling the speed of recombinant protein expression. To find a more suitable protein expression rate, the high- and low-strength promoter lacUV5 and lac were shuffled and recombined to yield the promoter variants lacUV5-1A and lac-1G. The results showed that only one base in lac promoter needs to be changed, and the A at the +1 position was changed to a G, resulting in a host of BL21 (DE3-lac1G), which successfully withstand the PCD of the host. The GDH activity at 43h was greatly increased from 37.5 U/mL to 452.0 U/mL. In scale-up fermentation, the new host was able to produce the model enzyme with a high rate of 89.55 U/mL/h at 43h, compared to the 3 U/mL/h of BL21 (DE3). Importantly, BL21 (DE3-lac1G) also successfully improved the production of other 10 enzymes. The engineered E. coli strain in the study conveniently optimizes recombinant protein overexpression by suppressing cell autolysis, and shows potential industrial applications.

Introduction

The pET system is a powerful tool for recombinant protein overexpression. It is based on host strains lysogenic for the DE3 prophage, with an integrated T7 RNA polymerase (RNAP) gene into the host genome, and cognate plasmids containing the T7 promoter. The transcription rate of T7 RNAP is about 8 times faster than that of the native *E. coli* RNAP (Jeong et al., 2009; Studier & Moffatt, 1986). BL21 (DE3) is arguably the most widely used protein production host. The promoter of the T7 RNAP gene in BL21 (DE3) is the lacUV5 mutant variant of the lac promoter, which is stronger than the original (Jeong et al., 2009).

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The reasoning behind the choice of these components for the production of proteins was straight-forward, based on the premise that more mRNA is beneficial for protein overexpression. To date, protein expression systems have been optimized widely by multiple approaches, including host reconstruction, expression vector redesign, and optimization of fermentation conditions (Costello et al., 2019; Li et al., 2016; Rosano et al., 2019).

However, BL21 (DE3) still cannot effectively produce certain proteins, especially toxic membrane proteins. Subsequently, the C41 (DE3) and C43 (DE3) strains were developed for membrane protein production. Studies have shown that C41 (DE3), in which the lacUV5 promoter was mutated into the weaker lac promoter, can effectively express toxic proteins (Schlegel et al., 2015). This mutant had a lower transcription rate of T7 RNAP, by which the toxic effect caused by overexpression of membrane proteins could be effectivity relieved (Kwon et al., 2015). Before, a derivative strain of BL21 (DE3), named Lemo21 (DE3), was engineered in which the activity of the T7 RNAP can be controlled by using the inhibitor T7 lysozyme (Wagner et al., 2008). Mutant56 (DE3) was isolated from a library of BL21 (DE3) variants, ant it was found that one amino acid was changed in T7 RNAP, which weakens the binding of the T7 RNAP to T7 promoter, thereby increasing membrane protein yields (Baumgarten et al., 2017).

In addition to lower expression of toxic proteins, BL21 (DE3) is also unable to effectively overexpress certain proteins that induced a physiological burden such as growth inhibition, cell lysis, or even death (Bhattacharya & Dubey, 1995). The production of this type of protein is normal during the early fermentation stage, but cells suffer autolysis at the later fermentation stage. For example, the presence of penicillin acylase inclusion bodies inhibited cell growth and caused serious cell lysis, so that approximately 76% of penicillin acylase was found in the extracellular medium (Narayanan et al., 2008). This phenomenon caused the fermentation time to be shortened, which ultimately reduced the yield of recombinant protein. Previous studies indicated that autolysis is not a direct result of the amount of the heterologous protein (Spada et al., 2002), but rather the global stress response induced by recombinant gene transcription or translation (Hoffmann & Rinas, 2004). Recently, many studies suggested that recombinant gene transcription, the mRNA level or even the speed of translation are the major causes of the growth inhibition or metabolic collapse (Li & Rinas, 2020). For example, under certain culture conditions, the accumulation of GFP can affect the growth of *E. coli* , but the effect could not be alleviated by removing the RBS of GFP (Mittal et al., 2018). In some cases, if the codons encoding the same amino acid in the same recombinant protein are different, the effect on inhibiting cell growth will also be different (Mittal et al., 2018; Natalie et al., 2015). However, the molecular mechanisms of this apparent RNA toxicity are presently unclear.

The cell lysis may be remedied by lowering expression, decreasing the growth temperature, or reducing target gene promoter activity. However, it is more meaningful and convenient for industrialization to construct a strong expression host that is intrinsically resistant to autolysis. In this study, an important industrial enzyme, GDH (EC:1.1.1.47), was applied as reporter protein. Blocking the known PCD pathway and controlling the speed of protein expression was used to suppress PCD, which promises to solve the urgent problems of industrial protein production.

2. Materials and methods

2.1 Construction of strains and plasmids

All plasmids used in this study are listed in Supporting information Table S1, and the primers are listed in Table S2. *E. coli* strain DH5α was used as the cloning host. The GDH plasmids variants with site-directed mutations and deletions of the RBS site (AAGGAG) were constructed using megaprimer PCR. The modified pET plasmids with RK2 replicon were constructed by standard restriction cloning based on Gibson assembly. All mutant strains were generated from BL21 (DE3) using CRISPR-Cas9 technology (Jiang et al., 2015). The template DNA with 1,000bp homologous arms was prepared by PCR. Strains harboring the pCas plasmid were transformed with the pTarget plasmid and the template DNA by electroporation (1.85 kV, 200 Ohm, 25 uF) and regenerated for 1 h in 1 mL LB medium at 37 . The mutants were confirmed by colony PCR. Correct colonies were then screened and the plasmids cured.

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2.2 Culture conditions

The *E. coli* strains was grown in Luria Bertani (LB) or Terrific Broth (TB) medium at 37 , supplemented where necessary with 100 μg/mL kanamycin (Kan). Flask fermentation was performed as follows: *E. coli* BL21 (DE3) harboring pET24a-GDH was grown in 3 mL of LB liquid medium overnight at 37. Then, 300 uL of the resulting culture were used to inoculate 30 mL of Terrific Broth (TB) medium in a 250ml shake flask. Cells were cultured at 37 to an OD_{600} of 2-4, at which point IPTG was added to a final concentration of 0.3 mM, and the fermentation was allowed to continue at 28 for an additional 40 h.

The GDH expression strains were first pre-cultured at 37 , 220 rpm for 16 h seeded into a 500 mL shake flask containing 100 mL of TB medium, and cultured at 37 °C, 220 rpm for 5 h. In the scale-up fermentation experiments, the resulting 100 mL seed culture was used to inoculate a 7.5-L fermenter containing 4 L of TY medium (Tao et al., 2014) at 37 at an aeration rate of 2.5 vvm. Agitation was steadily increased to a maximum of 800 rpm to maintain 20% dissolved oxygen (DO). When the DO further increased to more than 60%, 400 g/L glycerol, 50 g/L yeast extract, and 25 g/L tryptone were added into the medium, and the fermentation was continued at 800 rpm at an aeration rate of 2.5 vvm. When the OD_{600} reached 20, 0.2 mM IPTG was added to the medium and the cells were allowed to express the target protein at 28 for 43 h.

2.3 Scanning electron microscopy

BL21 (DE3) and BL21 (DE3-lac1G) cells harboring the pET24a-GDH expression vector were cultured as described above, and samples were collected at 24h and 43h, respectively. Samples were fixed overnight at 4°C in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) and then stained with 2% OsO4 in the same buffer. Then, samples were dried at critical point and coated with platinum. Observations were made using a Tecnai FEG ESEM Quanta 200 microscope (FEI Corp, USA), and images were recorded using SIS iTEM software (Olympus Corp, Japan).

2.4 GDH activity assay

To measure GDH activity, cells from 1 mL of fermentation broth were collected by centrifuging at 1,500 ×g for 10 minutes at 4. The culture supernatant was discarded, and the cells were resuspended in 1 mL of 100 mM Tris-HCl (pH 7.5). The cell suspensions was lysed by sonication on ice: 200 W, working 3 s, interval 5 s for 40 times, to yield the crude enzyme solution. The reaction mixture contained 100 mM glucose, 100 mM Tris-HCl (pH 7.5), 2 mM NAD^+, and an 8000-fold dilution of crude enzyme solution. The enzyme activity was assayed at 25 , by recording the increase of the absorbance at OD_{340}. The enzyme activity was calculated using the formula U/mL = [ΔA/min] × [1/ε] × 8000 (ε = A/cL = 6.402 mL / (mol * cm)) (Bucher et al., 1974).

2.5 SDS-PAGE analysis

The cells from 1 mL of fermentation broth were collected by centrifugation at 12000rpm for 10 min, and the supernatant was discarded. The cells were resuspended in 1 mL of 20 mM Tris-HCl buffer (pH 7.5) and disrupted using an Ultrasonic Cell Disruptor (operating for 5s, pausing for 5s, 40 times). The cell lysate was centrifuged at 4 °C, 12000 rpm for 10 min, and the soluble protein remained in the supernatant. An 80 μL aliquot of the supernatant was mixed with 20 μL of 5×loading buffer, and heated in a 100 °C metal bath for 10 min. The final protein samples were used for SDS-PAGE. Samples corresponding to 0.15 OD (10-well gel) or 0.075 OD (15-well gel) were added to the corresponding protein gel wells, and powered on until the electrophoresis was complete.

2.6 Quantitative real-time PCR

The relative transcription of genes was evaluated by qRT-PCR using the StepOnePlus Real-Time PCR System (Applied Biosystems, USA). Specific primers for qRT-PCR were designed to bind inside the T7RNAP gene, and amplify a fragment of approximately 220 bp. Total RNA was extracted using the Total RNA Isolation Kit (Blood/Cultured Cell/Fungus) (GeneDireX, Germany). The cDNA was produced using the SuperScript III First-Strand Synthesis System (Invitrogen USA). An aliquot containing 200 ng of cDNA was

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subjected to qRT-PCR using the EvaGreen qPCR System-ROX I (GeneDireX, Germany). The reaction (20 μL) contained 10 μL of SsoAdvanced Universal SYBR Green Supermix (BioRad, USA), 20 μM primers , 10 ng DNA, and adequate H_2O. Reactions were performed in white 96-well PCR plates (BioRad) and measured on a CFX384 real-time PCR detection system (BioRad) using the following thermal cycling parameters: 1 cycle, 95 ° C, 2 min; 40 cycles , 95 ° C, 10 s, 59 °C , 20s, 72°C , 20s; terminal melt-curve analysis (59-95 °C in 0.5 °C / 5 s increments). G6PDH was used as the endogenous reference gene to calculate the relative mRNA expression via the 2^{-T}method.

3 Results

3.1 GDH overproduction triggers autolysis

GDH is an important industrial enzyme, but high level production of GDH in BL21 (DE3) still presents a great technical challenge. In this study, the GDH activity suddenly decreased from 217 U / mL at 31h to 37.5 U / mL at 43h (Fig. 1A). Therefore, the fermentation time must be shortened, making it impossible to continue fermentation to obtain higher yields. Moreover, the OD in the later fermentation stage also showed a decline, indicating that autolysis may have caused cell death and a release of GDH into the extracellular medium. As shown in Fig. 1B, the SDS-PAGE showed a reduction of the intracellular heterologous protein, while there was a lot of the target protein in the extracellular medium. In order to investigate if autolysis was caused by the enzyme activity of GDH, the loss-of–function mutant $GDH_{Tyr253Cys}$was constructed (Makino et al., 1989), and the enzyme activity was decreased from 214.3 U / mL to 23.1 U / mL. However, the SDS-PAGE results showed that the mutant $GDH_{Tyr253Cys}$ still caused autolysis (Fig. 1C), which suggested that the autolysis was not related to enzyme activity. In addition, a mutant plasmid without RBS site was constructed to investigate if autolysis was caused by transcription rather than translation. The results showed that the cell growth was inhibited even after removing RBS site (Fig. S1), but no autolysis observed, which suggested that the intense burst of mRNA synthesis by T7 RNAP also had some toxic effects on *E. coli* . Furthermore, the membrane structure of *E. coli* cells during lysis due to GDH overexpression was visually inspected by means of scanning electron microscopy (SEM). The images showed extensive membrane elongation and shrinking (Fig. 1D), which confirmed the phenomenon of autolysis. Importantly, when amide hydrolase, Cephalosporin C acylase, formate dehydrogenase, R-carbonyl reductase, L-lactate dehydrogenase, L-2-hydroxyisocaproate dehydrogenase, L-phenylalanine dehydrogenase, secondary alcohol dehydrogenase and other 10 enzymes were individually overexpressed each also induced autolysis of BL21 (DE3). Therefore, solving the problem of autolysis in this workhorse strain is of great importance for industrial production.

3.2GDH expression resulted in programmed cell death

The combination of deceased OD and concomitant phenotypic changes provided convincing evidence that autolysis induced by GDH overexpression may be caused by triggering of a programmed cell death (PCD) pathway. The three major PCD pathways in bacteria are apoptotic-like death, mazEF-mediated death, and the cidA/lrgA holin-antiholin system. The RecA meditated apoptosis-like death pathway is thought to be activated by the SOS response (Lee et al., 2019b). The cidA/lrgA loci are regulators of the holin-antiholin system in *Staphylococcus aureus* , and the homologous locus of yohJK locus of *E.coli* was considered to have the same effect in *E. coli* (Dewachter et al., 2015). As shown in Fig. 2A, mRNA expression analysis showed that the expression of recA, mazEF, and yohJK at 43h was increased by 2.5-3.6 folds over their respective expression at 24h, which implied that autolysis could be triggered by PCD. To inhibit PCD mediated by known pathways, corresponding single and multiple deletion mutants were constructed. As shown in Fig. 2B, the $^{r}ecAstraincanexhibitedreductionoftheextracellularfractionofGDHenzymeactivityfrom90to65\%, butitdidnotmarkedlyin$
$mazEF-andyohJKdidnotalleviatethePCD, suggestingthatPCDinducedbyGDHoverproductionwasnottriggeredbytherepo$

3.3 Development of the BL21 (DE3-lac1G) strain that is resistant toPCD

The autolysis of DE3 strains may be controlled by various fermentation strategies, but obtaining a strong expression host that is intrinsically resistant to PCD is more meaningful and convenient for industrialization. As a common solution, C41 (DE3) was chosen as expression host, and the results showed that the GDH

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activity was increased from 173.7 U / mL at 24h to 207.3 U / mL at 43h (Fig. S2). It is important to note that there was no significant autolysis in the C41 (DE3) strain, although there was no great increase in GDH activity. Compared to BL21 (DE3), an important difference of the C41 (DE3) strain is the exchange of lacUV5 into a weaker lac promoter. In fact, the lacUV5 and lac (also named lac-1A) promoters differ only in two places and three points, so other two different promoter variants lacUV5-1A and lac-1G was obtained by shuffling and recombining of lacUV5 and lac-1A (Fig. 3A). Interestingly, the GDH activity increased significantly in the new strain BL21 (DE3-lac1G), resulting in 452.0 U / mL enzyme activity at 43h, which was about 12-times higher than that of BL21 (DE3) (Fig. 3B). However, the enzyme activity of the BL21 (DE3-1A) strain was lower than that of the starting strain BL21 (DE3). It is worth noting that although the enzyme activity of BL21 (DE3-lac1A) was low, there was no decrease of enzyme activity (Fig. 3B). More importantly, a very low extracellular GDH activity was obtained in BL21 (DE3-lac1G) strain (Fig. 3C), which implied that an appropriate T7 RNAP expression level was realized using the lac-1G promoter. Further SEM images indicated that the membranes of BL21 (DE3-lac1G) cells were smooth and robust at 43h (Fig. 3D), indicating that no PCD occurred in this new host. In the scale-up fed-batch fermentation, the GDH activity of BL21 (DE3) peaked at 31h and remained nearly unchanged thereafter, while that of BL21 (DE3-lac1G) was enhanced during the entire fermentation period, reaching yields of up to 3142.4 U / mL (Fig. 4). Notably, with increasing culture time, the productivity of GDH in BL21 (DE3) and BL21 (DE3-lac1G) followed opposite trajectories. In the BL21 (DE3) strain, the highest productivity was obtained at 7-19h, and then declined to only 3 U/mL/h at 31-43h. By contrast, the productivity of BL21 (DE3-lac1G) was increased constantly, and reaching 77.68 U/mL/h at 43-50h.

3.4 Analysis of key factors for controlling autolysis

To investigate whether the changes in the transcription level of T7 RNAP caused the difference in protein expression, the transcription levels of 4 promoters were measured by qRT-PCR. As shown in Figure 5A, the strength of the 4 promoters were lac-1A <lac-1G<lacUV5<lacUV5-1A. The transcription level of T7 RNAP from $P_{lac\text{-}1G}$ was only 16.4% that of P_{lacUV5}. Consequently, the former maintained GDH expression at a slightly lower level, which might be the key reason for suppression of PCD. In a previous study, the expression capacity of T7 RNAP within a wide range was realized by designing RBS, which precisely regulate the expression of recombinant proteins (Liang et al., 2018). However, compared to $P_{lac\text{-}1A}$, the relative transcription level of $P_{lac\text{-}1G}$ was increased by 2.05 times, which resulted in faster GDH accumulation than in BL21 (DE3-lac1A), which finally led to the highest performance. Interestingly, the transcription level of the lacUV5-1A promoter showed a 2.68 folds increase, indicating that a strong promoter is not conducive to the expression of GDH and may be the key reason for PCD.

In addition to controlling the expression level of T7 RNAP, we investigated if it could also be prevented by modifying the copy number of the plasmid carrying the target gene. High copy number plasmid for protein production puts extra metabolic load on host cells, resulting inhibition of cell growth and plasmid instability (Bentley et al., 2009). In a previous study, sequence homology between CoIE1 RNA I/ RNA II and tRNAs was abolished to keep the plasmid copy number constant, then metabolic activity can be prolonged (Grabherr et al., 2002). Based on this, the replicon of the pET plasmid (CoIE1) with 15 copies was replaced by the RK2 replicon with 5 copies. Then, the BL21 (DE3-lac1G)-R2K and BL21 (DE3)-R2K strains with combinations of "strong + low copies" and "weak + low copies" were obtained, respectively. As shown in Fig. 5B, although the GDH activity of BL21 (DE3)-R2K was higher than that of BL21 (DE3) -colE1, it was only 51.62% that of the BL21 (DE3-lac1G)-colE1. The "weak + low copies" group exhibited much lower enzyme activity than the other groups. Overall, controlling the expression level of T7 RNAP is more effective than controlling the copy number of the plasmid, implying that T7 RNAP plays the role of the lysis "switch" in the pET system for recombinant protein overexpression (Fig. 5C).

3.5 Stability and versatility of BL21 (DE3-lac1G) strain

Stability and versatility are key indicators for investigating the quality of protein expression hosts. As shown in Fig. 6A, overall, the ration of plasmid-carrying cells in BL21 (DE3) continued to decline with increasing fermentation time, reaching a low of 40% at 36h and decreasing further to 0 at 43h. By contrast, the ratio of

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plasmid-carrying cells in BL21 (DE3-lac1G) remained about 96.5% at 43h, and about 49% of the cells maintained enzyme activity at normal levels (˜ 350 U / mL) (Fig. 6B). Therefore, the stability of the new protein expression host is much higher than that of the parent strain. Moreover, the SDS-PAGE images showed that BL21 (DE3-lac1G) is also resistant to autolysis that is triggered by a further 10 tested recombinant proteins, including amide hydrolase, cephalosporin C acylase, formate dehydrogenase, R-carbonyl reductase, L-lactate dehydrogenase, L-2-hydroxyisocaproate dehydrogenase, L-phenylalanine dehydrogenase, secondary alcohol dehydrogenase, D-2-hydroxyisocaproate dehydrogenase, S-carbonyl reductase (Fig. 7).

Discussion

In the last decades of the 20$^{\text{th}}$ century, *E.coli*BL21 (DE3) has become the preferred host for recombinant protein production. However,some recombinant proteins may impose a high metabolic burden or lead to toxicity in the host cell, which may result in reduced growth rate, low final cell density, and even cell death (Bhattacharya & Dubey, 1995). After 24h, GDH expression inhibited cell growth and induced severe autolysis (Fig. 1A and B). For the expression of toxic membrane protein, a common strategy is to decrease the expression of the toxin protein by governing the expression of T7 RNAP, such as C41 (DE3) and Lemo21 (DE3). Notably, the C41 (DE3) strain weaken the lacUV5 promoter by recA-dependent recombination with the lac promoter (Susan et al., 2015), indicating that the strength of the lac promoter may not be optimal. However, the C41 (DE3) strain could not effectively improve the expression of GDH compared to BL21 (DE3), which implied that the toxic effect of GDH is different from that of toxic membrane proteins. Most importantly, the autolysis phenotype appeared during the expression of a variety of proteins, which necessitates shortening the fermentation time, and eventually reduces the protein yield. The host mutation of BL21 (DE3-lac1G) changes one base in the lac-1A promoter, which had an enormous positive impact on autolysis, ie., a high rate of 89.55 U/mL/h was obtained at 43h in comparison of that of BL21 (DE3) with 3 U/mL/h. Taken together, our results proved that the new strain BL21 (DE3-lac1G) strain can effectively suppressing PCD and maintain plasmid stability, which will make it a popular host for protein production, especially for proteins requiring a longer fermentation time or maturation with after processing.

The production of foreign proteins often imposes a metabolic burden on the host by triggering local and global cellular stress responses (Bhattacharya & Dubey, 1995). In particular, this metabolic burden often manifests as an increase of the energy demand or maintenance energy requirement such as amino acids, ribosomes or other precursors (Mairhofer et al., 2013). The metabolic burden on host cells can be improved by making right choices for promoter (Pasini et al., 2016), plasmid copy number (Flores et al., 2004), and removal of codon bias (Rahmen et al., 2015). Moreover, optimizing environment conditions also can reduced metabolic burden, such as addition of amino acids or using complex medium (Fong & Wood, 2010). As a first measure, if the growth rate of recombinant strain is inhibited, then two causes may explain the phenotype: gene toxicity and basal expression of the toxic mRNA/protein. Protein toxicity refers to the toxicity of the target protein itself, such as its enzyme activity or cell damage due to misfolded aggregates (Binepal et al., 2012). On the other hand, studies have reported that excessive T7 RNAP itself can be lethal to cells (Davanloo et al., 1984). Moreover, it was found that the toxicity caused by mRNA may be neither related to plasmid abundance nor to the abundance of the encoded mRNA (Mittal et al., 2018). Previous studies also proved that the toxicity of certain gene was only dependent on transcription but independent of protein translation (Li & Rinas, 2020). In this study, a GDH mutant with dramatically reduced activity and a construct without RBS proved that autolysis was not caused by the activity or amount of protein, so it is likely that the autolysis is induced by certain mRNA elements. In this study, the T7 RNAP expression from lac-1G promoter was found to be higher than from lac-1A promoter, but lower than from lacUV5. Consequently, the rate of protein overexpression in BL21 (DE3-lac1G) was manipulated at a will level, not only transcription with a higher level but also no more than the host tolerance during the whole fermentation stage. A recent study of a plasmid-driven T7 (PDT7) system also suggested that high expression of T7 RNAP affected cell metabolism and led to toxicity and instability (Tan & Ng, 2020). In fact, the T7 RNAP itself could be not toxic, but when it combined with a strong promoter, it can induce severe growth defects. A excessively strong T7 RNAP system robbed energy from the basal metabolism, which is the major reason of T7 RNAP toxicity (Tan & Ng, 2020). By inhibiting endogenous RNAP or reducing parts of non-essential

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proteome production, it is possible to balance cell growth and recombinant protein production on resources allocation (Kim et al., 2019). Recently, the evolved T7 phage RNAP inhibitor Gp2 was used in BL21 (DE3) to decouple recombinant protein production from cell growth, which enhanced protein yields up to 3.4-fold (Stargardt et al., 2020). Moreover, the resources can be selectively allocated for transcription or translation of target genes by orthogonal molecular elements (Darlington et al., 2018; Segall-Shapiro et al., 2014), which were beneficial to reduce the metabolic burden of the host cells. This study proved that the key to regulating autolysis and protein overexpression is controlling the expression of T7 RNAP, which plays the role of the main on-off "switch" in the pET system. Moreover, controlling the rate of transcription of T7 RNAP can mitigate the metabolic burden effectively and easily. The new host strain of BL21 (DE3-lac1G) can effectively produce recombinant protein without affecting growth.

Under excessive stress, the repair mechanisms will be overwhelmed and cells will undergo programmed cell death (PCD). This program offers no direct advantage to individual cells, but could benefit its siblings by releasing nutrients for other cells in the colony or preventing the spread of viruses (Tanouchi et al., 2013). The death involved PCD pathway is meditated by an intracellular program (Nagamalleswari et al., 2017), mainly including the toxin-antitoxin system, holin-antiholin system and ALD pathway. The membrane integrity of BL21 (DE3) was impaired when overexpressing GDH. More importantly, the expression level of PCD markers including recA, mazEF, and yohJK were showed to be up-regulated at 43h compared to 24h (Fig. 2A), which suggested that BL21(DE3) suffered PCD at 43h. In general, deletion of the key genes involved in the PCD pathway can restore cell viability. For example, periplanetasin-2 was proven to induce apoptosis-like death in*E.coli* according to physiological changes, which was proven when the antibacterial activity of periplanetasin-2 was decreased by deletion of recA (Lee et al., 2019a). However, deletion of three key genes did not restore viability in the GDH production process, indicating that cell death of BL21 (DE3) does not proceed only through these three general bacterial PCD pathway. In the new strain BL21 (DE3-lac1G), the lower protein expression rate did not exceed the cellular metabolic capacity, and therefore could not induce PCD. Nevertheless, the common characteristics of proteins that cause PCD and how it is promoted needs further study.

In the future, this novel autolysis system can be used in secretory protein production. The production of extracellular proteins has distinct advantages, such as simplifying the disruption of the cell wall and purification processes (Su et al., 2013). Previous studies applied holins and endolysins to promote cell lysis, combined with various inducible promoters to prevent cell lysis before sufficient cell growth (Choi & Lee, 2004). However, expression of lytic proteins still uses valuable cellular energy resources. Inspired by this study, the promoter of T7 RNAP can be designed to let cells overexpress too much of the target protein at a desired time-point induce autolysis, so that the host produces the target protein from beginning to end, without relying on exogenous lysing proteins. In addition, four promoters with different strength can form a series of protein expression hosts with different T7 RNAP expression levels, providing a variety of host choices. Moreover, the promoter strength of lacUV5-1A is 2.68 times higher than that of the strong promoter lacUV5, which indicated that lacUV5-1A promoter can satisfy the requirements of high expression of target genes in metabolic engineering in the future.

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Competing interests

The authors declare that they have no competing interests.

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Supporting Information

Table S1: Strains and plasmids used in this work.

Table S2. Primers used in this work.

Figure S1. OD600 analysis of BL21 (DE3) with expression vector without RBS.

Figure S2. Comparison between BL21 (DE3) and C41 (DE3) for GDH activity.

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Figure captions

Figure 1 . GDH overproduction triggers autolysis of BL21 (DE3). (A) GDH activity and OD were monitored during the whole fermentation period. (B) SDS-PAGE results of intracellular and extracellular protein in BL21 (DE3) at 46h. The white rectangle was used to indicate the GDH expression band. (C) SDS-PAGE results of intracellular and extracellular protein in BL21 (DE3)-GDH and BL21 (DE3)-GDH-mutant strain at 31h. (D) The cell membrane changes in BL21 (DE3) strain between 24h and 43h were observed by SEM. The red arrow indicates the protrusions or shrinking of cell membrane. Values and error bars represent the means and the deviations from triplicate experiments.

Figure 2. PCD was found to be induced in the process of GDH overproduction, and GDH activity was analysis after blocking the reported PCD pathway. (A) Quantitative real-time PCR results at 24h and 43h for the three key genes (*recA* , *mazEF* and *yohJk*) involved in the PCD pathway. (B) The changes of intracellular and extracellular GDH activity was measured at 24h and 43h in BL21 (DE3) as well as $^{r}ecA, ^{y}ohJK, ^{m}azEF, and ^{r}ecA + yohJK + mazEFderivatives. Valuesanderrorbarsrepresentthemeansandthedeviationsfromtriplicateexperiments.$

Figure 3 . BL21 (DE3-lac1G) greatly reduced autolysis and enhanced GDH overproduction. (A) Sequence variation between the four types of promoters. Substitutions are marked in red. (B) The extracellular GDH activity under four different promoters was measured during the entire fermentation period. (C) Comparison between the BL21 (DE3) and BL21 (DE3-lac1G) for GDH activity and SDS-PAGE at 43h. (D) The cell membrane changes of BL21 (DE3-lac1G) strain between 24h and 43h was investigated by SEM. Values and error bars represent the means and the deviations from triplicate experiments.

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Figure 4. Comparison between BL21 (DE3) and BL21 (DE3-lac1G) for GDH activity and productivity in scale-up fermentation. Values and error bars represent the means and the deviations from triplicate experiments.

Figure 5. Performance assessment of the BL21 (DE3-lac1G) strain. (A) The relative mRNA expression of T7 RNAP under four different promoters. (B) The GDH activity changes under different combinations with weak/strong T7 RNAP expression levels and high/low plasmid copy numbers. (C) Model explaining the differences in behavior between BL21 (DE3-lac1G) and BL21 (DE3). Values and error bars represent the means and the deviations from triplicate experiments.

Figure 6. Plasmid stability test of BL21 (DE3-lac1G) strain. (A) The percentage of plasmid-carrying cells of BL21 (DE3)-PET24a (empty), BL21 (DE3) and BL21 (DE3-lac1G) was tested during the entire fermentation period. (B) Overview of stability determination during the fermentation (above), and the GDH activity of 45 clonds isolated at the end of fermentation (below).

Figure 7. The BL21 (DE3-lac1G) strain exhibited a superior overexpression capacity with 10 additional different heterologous proteins compared with the parental strain BL21 (DE3). SDS-PAGE with intracellular and extracellular proteins expressed in BL21 (DE3) and BL21 (DE3-lac1G) was showed and compared. The white rectangle indicates the target protein expression band.

Figure S1. OD600 analysis of BL21 (DE3) with expression vector without RBS. Values and error bars represent the means and the standard deviations of triplicate experiments.

Figure S2. Comparison between BL21 (DE3) and C41 (DE3) for GDH activity. Values and error bars represent the means and the standard deviations of triplicate experiments.

Figure 1

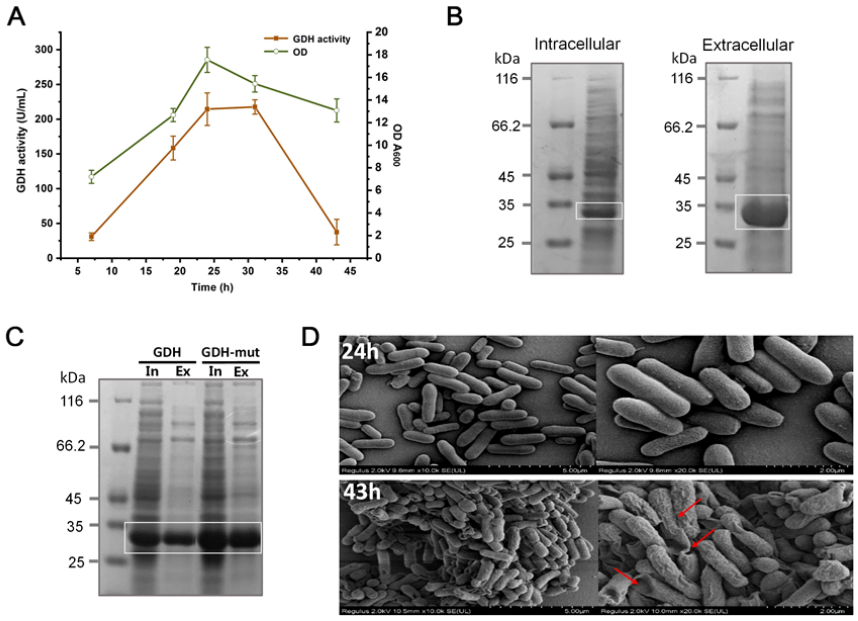

Figure 2

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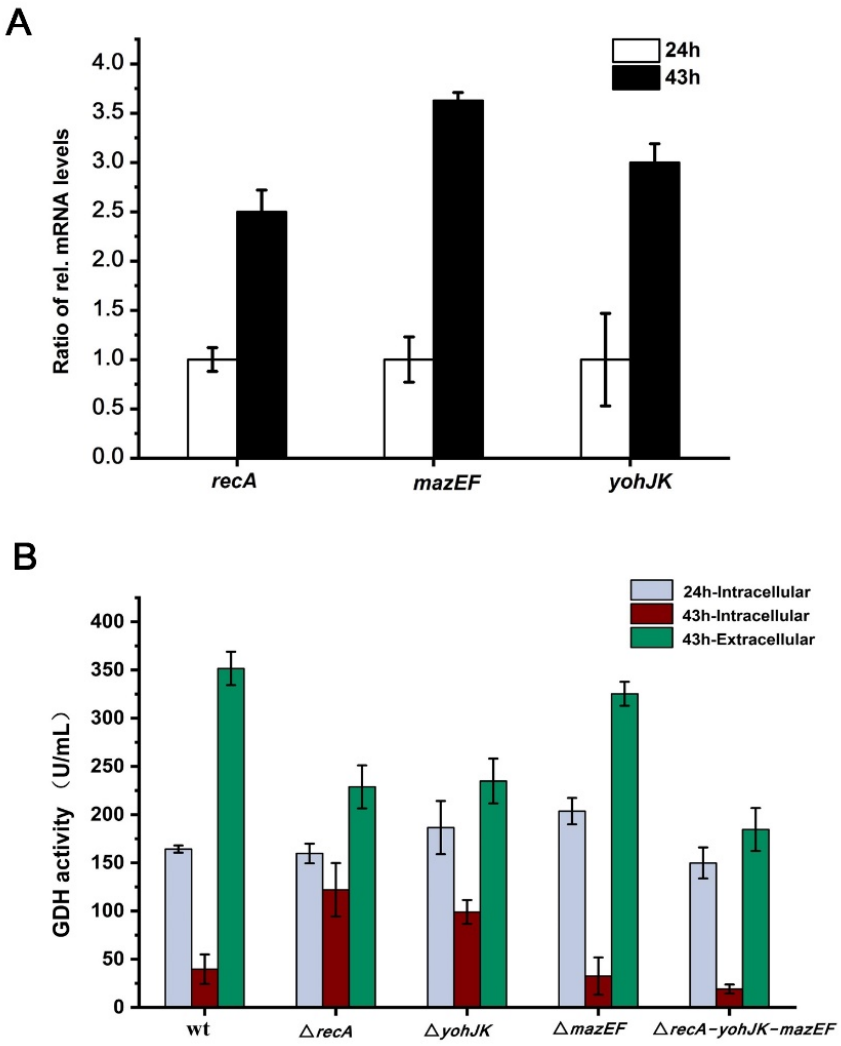

Figure 3

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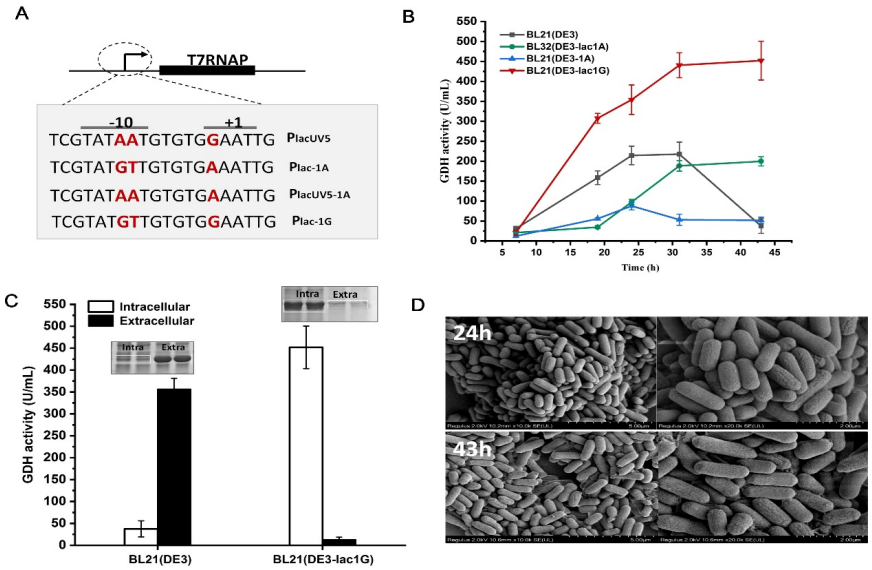

Figure 4

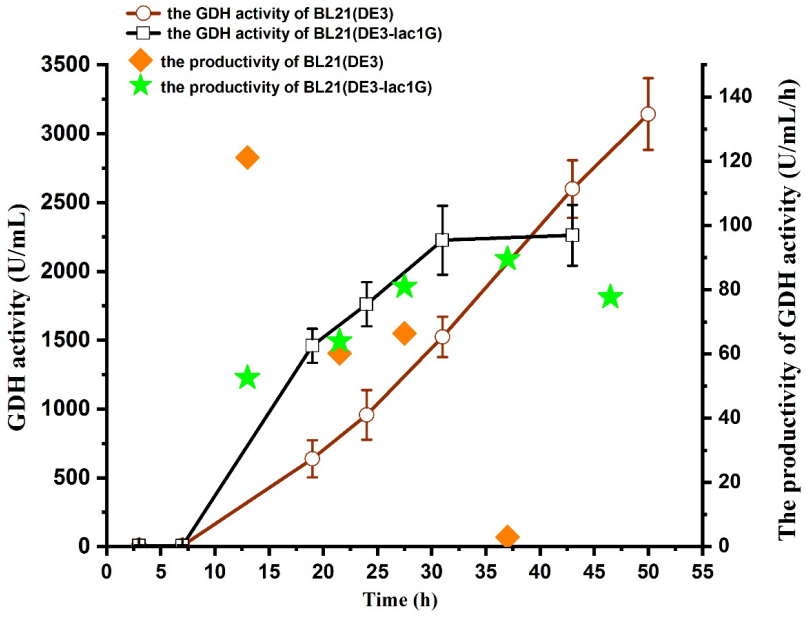

Figure 5

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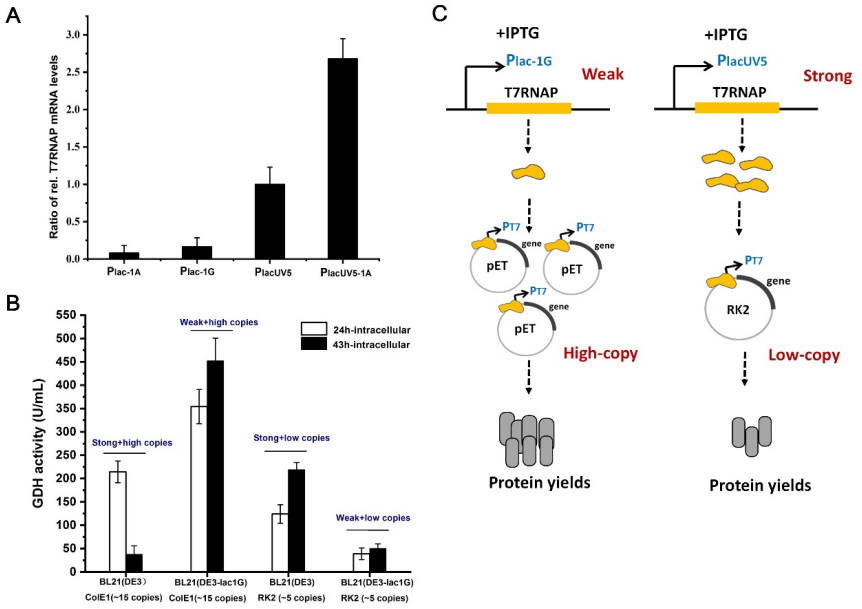

Figure 6

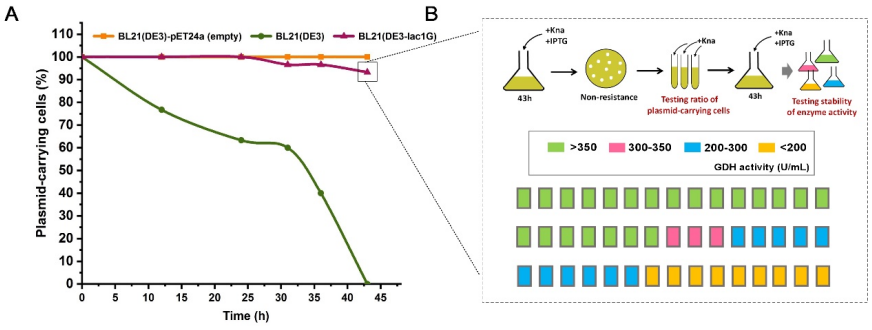

Figure 7

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