Regulating the T7 RNA Polymerase Expression in E. coli BL21 (DE3) to Provide More Host Options for Recombinant Protein Production

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Abstract

Escherichia coli is the most widely used bacterium in prokaryotic expression system for the production of recombinant proteins. In BL21 (DE3), the gene encoding the T7 RNA polymerase (T7 RNAP) is under control of the strong lacUV5 promoter (P_{LacUV5}), which produces more T7 RNAP than wild-type lac promoter (P_{LacWT}) to promote the production of recombinant proteins. However, there is a resource allocated limitation between cell growth and protein production when producing autolytic proteins or membrane proteins. T7 RNAP is the key factor to solve this problem. Hence, we replaced respectively P_{LacUV5} with other inducible promoters: arabinose promoter (P_{araBAD}), rhamnose promoter (P_{rhaBAD}), tetracycline promoter (P_{tet}) to optimize the production of recombinant protein by regulating the transcription level of T7 RNAP. Compared with BL21 (DE3), the constructed engineering strains had higher sensitivity to inducers, among which rhamnose and tetracycline promoters had the lowest leakage ability. In the glucose dehydrogenase (GDH) production, the engineered strains BL21 (DE3::tet) exhibited great biomass, cell survival rate and foreign protein expression level. In addition, these engineered strains had been successfully applied to the production of other membrane proteins, including E. coli cytosine transporter protein (CodB), the E. coli membrane protein insertase/foldase (YidC), and E. coli F-ATPase subunit b (Ecb). The engineering strains constructed in this paper provided more host choices for the production of recombinant proteins.

1. Introduction

Escherichia coli BL21 (DE3) and PET expression system are the most representative recombinant protein expression systems [1]. In BL21 (DE3), expression of the gene encoding the target protein, which is on the PET plasmid, is driven by the chromosomally encoded bacteriophage T7 RNA polymerase (T7 RNAP). The T7 RNAP specifically recognizes the T7 promoter and transcribes eight times faster than *E. coli* RNAP [2-4]. The gene encoding the T7 RNAP is governed by the isopropyl β-D-1-thiogalactopyranoside (IPTG) -inducible LacUV5 promoter (P_{LacUV5}), which is a strong variant of the wild-type Lac promoter (P_{LacWT}) [5]. The reasoning behind the choice of these components to create a protein overexpression system is straight-forward, the more mRNA is synthesized, the more protein can be produced.

However, this system is not suitable for the expression of all proteins. On one hand, the strong P_{LacUV5} will produce more T7 RNAP, which will occupy more resources for the production of recombinant protein, resulting in the limitation of resource allocation for cell growth and protein production [6, 7]. The expression level of T7 RNAP determines the production of recombinant protein in BL21 (DE3) [8]. Based on this, researchers had developed many methods to balance intracellular resource allocation, such as decoupling protein production from cell growth and changing promoter intensity. Stargardt et al. realized the decoupling of cell growth and protein expression by introducing the gene GP2 (an inhibitor of *E. coli* RNA polymerase), the resources in the cell flowed to the expression of T7 RNAP to produce the target protein when there was enough biomass [9, 10]. Recently, a gene growth switch based on CRISPRi was developed to achieve efficient production of target proteins by targeting genes related to cell growth and

DNA replication in *E. coli* [11]. Sun et al. constructed a non-autolytic strain capable of efficiently producing recombinant proteins by hybridizing the weak Lac promoter with the strong P_{LacUV5} [12]. On another hand, the P_{LacUV5} has a higher basic leakage expression, which cannot provide precise control of gene expression [13, 14]. The LacI mutant was developed, which only can bind to the lacO (Lac operator) and significantly reduced the basic leakage expression of P_{LacUV5} [15]. Furthermore, there is a disadvantage that the addition of the inducer IPTG will cause toxicity to the cells [13, 14]. To eliminate the toxicity of IPTG to cells, the BL21-Al<gp2> strain using arabinose promoter to drive transcription of the T7 RNAP was developed and used to successfully overexpress toxic proteins [9].

In this study, we constructed three engineering strains to regulate the transcription level of T7 RNAP and leakage expression of P_{LacUV5} through promoter engineering, which expands host strains for recombinant protein expression. Specifically, engineering strains were constructed by replacing respectively P_{LacUV5} with other inducible promoters: arabinose promoter (P_{araBAD}), rhamnose promoter(P_{rhaBAD}), tetracycline promoter(P_{tet}). Furthermore, the novel engineering strains were successfully applied to overproduce three membrane proteins, *E. coli* cytosine transporter protein (CodB), the *E. coli* membrane protein insertase/foldase (YidC), and *E. coli* F-ATPase subunit b (Ecb), which had been reported to be difficult to produce in *E. coli* strains (Masanari et al., 2006; Miroux & Walker, 1996; Wagner et al., 2007)(Masanari et al., 2006; Miroux & Walker, 1996; Wagner et al., 2007)[13,16,17]. This article has important research significance for improving the expression of recombinant protein.

2. Material And Methods

2.1 Bacterial Strains and Plasmid Constructions

All plasmids and recombinant proteins used in this study were listed in Table S1, and the DNA primers were listed in Table S2. The *E. coli* DH5α was used for plasmid construction, and BL21 (DE3) was applied for gene expression. All plasmids used to express recombinant protein are constructed on the basis of pET24a in this paper. The fragments plasmid skeleton obtained by PCR were linked by Gibson assembly. In the process of plasmid construction, the recombinant proteins expressed in cells was C-terminally fused to EGFP. The constructed plasmid was transformed into DH5a by chemical transformation method and the transformants were confirmed by colony PCR. Correct colonies were screened and the plasmids cured and plasmids were obtained by plasmid extraction kit.

2.2 Culture conditions

All *E. coli* strains were cultivated in Luria–Bertani (LB) medium which contained 10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl at 37 °C with constant shaking at 220 rpm, and added 2% agar if it was solid LB medium. The fermentation strains grown in Terrific Broth (TB) mediumat 28 °C with constant shaking at 220 rpm. The medium was supplemented with 50 μg/mL kanamycin (kan) or Spectacular (spec) according to the screening markers carried by intracellular plasmids.

Flask fermentation was performed as follows: tha newly constructed strains with different promoters harboring recombinant protein plasmids were grown in 3 mL of LB liquid medium overnight at 37 °C. Then, 300 μL of the resulting culture were used to inoculate 30 mL of TB medium in a 250 mL shake flask. Cells were cultured at 37 °C to an OD_{600} of 2–4, at which point Isopropyl β-D-1-thiogalactopyranoside (IPTG), L-arabinose, L-rhamnose (L-rha), anhydrotetracycline (aTc) were added to a final concentration of 0.3 mM, 10mM, 10mM, 100ng/mL respectively. The fermentation was allowed to continue at 28 °C for an additional 60 h. In the fed-batch fermentation process, added 10% fresh TB medium at 24 h and continued fermentation until the end.

2.3 Construction of engineered strains

In this paper, the four inducible promoters used included P_{LacUV5}, $P_{araBAD,}$ P_{rhaBAD} and P_{tet}. Plasmids pTarget-ara/pTarget-rha/pTarget-tet were obtained by placing different promoter sequences on gRNA plasmids respectively and the template DNA with 1,000 bp homologous arms was prepared by PCR. The primers of these promoters obtained by PCR were listed in Table S2. The first plasmid, pCas, was transformed into BL21 (DE3) strain. Then the gRNA plasmid with donor fragment was transferred into the above strain. The promoter controlling T7 RNAP in BL21 (DE3) genome was replaced by CRISPR/Cas9 technology. The correct transformants were confirmed by colony PCR and resistance markers. Plasmids were discarded in turn by the resistance marker in plasmid. We can obtained new strains which T7 RNAP was placed respectively under $P_{araBAD,}$ P_{rhaBAD} and P_{tet}.

2.4 Scanning fluorescence microscope and fluorescence intensity

The pET24a-EGFP plasmid was constructed by amplifying the EGFP fragment with primers EGFP-F and EGFP-R and cloning into pET24a, which was digested with Dph1 in advance. Plasmids were respectively transferred into newly constructed strains and original strains BL21 (DE3). To prepare competent cells, a transformant cultivated at 37 °C until the OD_{600} reached 0.6-0.8. The calcium chloride method was used to transfer the pET24a-EGFP plasmid into the host strain, followed by recovery at 37 °C for 1 h. Then, positive colonies were screened on LB solid medium with 50 μg/mL kan at 37 °C. One colony was randomly selected and pre-cultured in LB with antibiotic at 37 °C. The strains were cultured according to the method we described before fermented for 18 hours.

The cell growth was monitored by absorbance at 600 nm (OD_{600}) and a small amount of cells were observed under fluorescence microscope. 500 μL of cells was centrifuged at 13 000 rpm for 5 min and then resuspended in 500 μL of phosphate-buffered saline (PBS). One hundred microliters of the resuspensions were transferred to 96-well Black Optiplate 96F plates. The whole cell fluorescence was detected with a 485 nm excitation wavelength and 535 nm emission wavelength. All experiments were performed in triplicates.

2.5 Plasmid stability analysis

Samples were taken at 24, 36 and 48 h of fermentation, and diluted to the same OD using PBS. 100 uL of dilution liquid was coated on LB solid plate with 50 μg/mL kanamycin. After culturing for 12 h, 100 single colonies were picked and spotted on LB solid plate and LB solid plate containing 50 μg/mL kan respectively. After cultivation for 12 h at 37 °C, colonies growing on LB solid plate but not on LB with 50 μg/mL kan were interpreted that the plasmid has been lost. The plasmid stability was calculated as the number of samples with growth in LB with kanamycin/100× 100%.

2.6 Protein expression based on engineered strains

The recombinant proteins expressed in four strains was C-terminally fused to EGFP. Therefore, the expression of recombinant protein can be observed by fluorescence value. The processed samples were examined according to the method described above to calculate unit cell fluorescence value. All experiments were performed in triplicates.

3. Results

3.1 Construction and characterization of engineering strains

The constructed DNA expression cassettes of different promoters were integrated into the chromosome of *E. coli* BL21 (DE3) by CRISPR/Cas9 (Fig. 1a). And the engineering strains were respectively denoted as BL21 (DE3::ara), BL21 (DE3::rha), BL21 (DE3::tet).

First, the leakage ability of the promoter and the transcription level of T7 RNAP of the engineering strains were characterized by fluorescence intensity. As shown in Fig. 1b, under the condition of no inducer, there was obviously higher florescence intensity in original strain BL21 (DE3) than other three engineering strains, which proved that the P_{LacUV5} has a high basic leakage expression and cannot achieve strict regulation of gene expression. It can be seen that the P_{rhaBAD} and the P_{tet} had the lowest leakage ability. When inducer was added, the average fluorescence value of BL21 (DE3::rha) and BL21 (DE3::tet) reached 430,000 a.u, which was 2.11 and 2.0 times higher than that of BL21 (DE3), respectively. The same phenomenon can also be obtained in Fig. 1c. Compared with the engineered strains, the fluorescence image of strain BL21 (DE3) was the brightest without induction. After induction, the brightness of the fluorescent images of strains BL21 (DE3::rha) and BL21 (DE3::tet) were higher than that of strain BL21 (DE3) (Fig. 1c). In general, the P_{rhaBAD} was considered to be the best promoter, as it offers tight control of gene expression with negligible background expression.

3.2 Application of engineered host strains in GDH production

Studies had shown that when BL21 (DE3) overexpressed glucose dehydrogenase (GDH), an autolysable protein, severe cell autolysis was induced, resulting in low protein production [12]. Therefore, we investigated whether the three engineered strains can improve the expression of GDH through fluorescence intensity (Fig. 2a). In the first 24 hours of GDH production, the growth status and protein production of BL21 (DE3) strain were optimal. However, the growth of the BL21 (DE3) strain slowed down

and the biomass decreased drastically, while the cell biomass and the fluorescence intensity of the three engineered strains continued to rise from 24th to 36th. In particular, at the 36th hour of protein production, the fluorescence intensity of strain BL21 (DE3::ara) was 1.57 times that of BL21 (DE3), the fluorescence intensity of strain BL21 (DE3::rha) was 1.37 times as high as that of strain BL21 (DE3).

Furthermore, the intracellular stability of the expression plasmid GDH in the later stage of fermentation was tested (Fig. 2b). As shown in Fig. 2b, the ratio of plasmid-carrying cells in BL21 (DE3) continued to decline with increasing fermentation time, reaching a low of 2.17% at 36 h. By contrast, the ratio of plasmid-carrying cells in BL21 (DE3::ara), BL21 (DE3::rha) and BL21 (DE3::tet) remained respectively about 100%、99% and 100% at 36 h. More importantly, the stability of the engineered host strains were much higher than that of the parent strain. Among them, the engineered strain BL21 (DE3::ara) was more effective in expressing GDH, which was probably ascribe to its tolerance to GDH overexpression by regulating the resource allocation between cell growth and protein production. The survival rate of the three engineered strains were still high, and the expression plasmid existed stably in the cell, which indicated that the yield of autolytic proteins can be further improved by prolonging the fermentation time.

3.3 Application of engineered host strains in membrane proteins production

The application of engineered strains in the expression of three membrane proteins was further investigated, and the results were shown in Fig. 3. In the production of membrane protein Codb, after adding the inducer, the fluorescence intensity of BL21 (DE3) strain reached 36,461 a.u at 12 h, which were 11.8, 3.0 and 6.8 times higher than those of other three engineering strains at the same stage (Fig. 3a). The fluorescence intensity of the BL21 (DE3) strain dropped sharply, while the fluorescence intensity of the three engineered strains continued to increase steadily after 24 h. Among them, The fluorescence intensity of the BL21 (DE3::rha) strain reached 10,870 a.u., the cell biomass has little change, and the average ratio of plasmid-carrying cells was about 98% (Fig. 3b). The BL21 (DE3::rha) strain was considered to be the most suitable host strain for Codb production. Similarly, the fluorescence intensity of strain BL21 (DE3) first increased and then decreased for production of Ecb, while the three constructed strains has been showing an upward trend (Fig. 3c). At 60 h, the fluorescence of BL21 (DE3::rha) and BL21 (DE3::tet) were 6913a.u. and 5711a.u., which were 2.3 and 1.6 times higher than those of BL21 (DE3) strains at the same atagr, respectively. The ratio of plasmid-carrying cells in three engineered strains remained about 98% at 48 h (Fig. 3d). BL21 (DE3::rha) was considered to be the best host choice for Ecb production. In the production of Yidc, the fluorescence intensity of BL21 (DE3::tet) reached 3519 a.u. at 18 h, which was 2.7 times that of the BL21 (DE3) strain (Fig. 3e), and 96% of the plasmids were stably existed and functioning (Fig. 3f). The BL21 (DE3::tet) strain was more suitable for Yidc production. In short, the best host for producing different membrane proteins was different, which was contributed to requirements for T7 RNAP of different membrane proteins. Therefore, the three engineered strains had been successfully applied to the production of membrane proteins.

3.4 Fine regulation of the concentration of inducer can further improve target protein production

It had been reported that the concentration of the inducer has a great influence on the final expression yield of the protein [15, 18]. In order to further improve the application potential of the engineered strains, the production capacity of the three engineered strains and BL21 (DE3) strain were investigated by adding different inducer concentrations on the basis of the previous research. The addition concentration of IPTG was 0.1 mM, 0.3 mM and 0.5 mM respectively. The concentration of L-arabinose was 2.5 mM, 10 mM, 20 mM respectively. The concentration of L-rhamnose (L-rha) added was 2 mM, 10 mM, 40 mM respectively. And anhydrotetracycline (aTc) was added at 250 ng/mL, 1000 ng/mL, 3000 ng/mL respectively.

The fig. 4 showed the fluorescence intensity of four engineered strains producing different recombinant proteins mentioned above under different concentrations of inducers at 36h. The fluorescence intensity of the three engineered strains were all higher than BL21 (DE3), When the two recombinant proteins GDH and Yidc were fermented (Fig. 4a, d). Compared with BL21 (DE3), the unit cell fluorescence intensity of the three engineering strains were lower when the other two proteins were produced (Fig. 4b, c), which may be the result of the decrease of cell biomass in the later stage of fermentation. It can be concluded from the results above that the concentration of inducer was closely related to protein expression. And the concentration of inducer required for optimal protein expression was different when producing different proteins. It was proved through this experiment that the expression of recombinant protein can be more finely regulated by changing the concentration of inducer.

Discussion

It is acknowledged that the pET expression system is a powerful tool for the production of recombinant proteins. However, it has been shown that T7 RNAP-mediated expression of genes encoding membrane proteins is usually toxic, which causes damage to cell [13]. There is another explanation that one of the reasons for this phenomenon was that the stronger P_{LacUV5} produced a large amount of T7 RNAP, which was toxic to cells [19, 20]. Other studies had shown that this phenomenon was caused by the resource allocated limitation between cell growth and protein production, rather than the toxicity of T7 RNAP. Wagner et al. revealed that mutations in the promoter governing expression of T7 RNAP were key to the improved membrane protein overexpression characteristics of the Walker strains by using a combination of proteomics and genetics [21, 22]. Therefore, T7 RNAP inhibitor T7 Lys was used to dampen T7 RNAP activity to improve membrane protein production [23]. In order to further improve the production of membrane proteins, this study adjusted the transcription level of T7 RNAP by replacing the promoter controlling T7 RNAP, and further adjusted the resource allocation of cell growth and protein expression to optimize protein expression. The fluorescence intensity of BL21 (DE3) strain decreased obviously, and 97% of cells did not contain expression plasmid when the recombinant protein mentioned above were produced after 24h (Fig. 2b, 3b, 3d, and 3f). On the contrary, the three engineered strains continued to rise, 98% of the cells still existed stably and played a role even after 48 hours, which proved that selecting suitable promoters can reduce the growth burden of host cells [24]. Previous studies had shown that P_{rhaBAD} controlled T7 RNAP to be placed on PET plasmid for gene expression [15], but this had the

disadvantage that the plasmid can not exist stably in the cell and the copy number was limited. In this study, we modified the promoter controlling T7 RNAP on BL21 (DE3) chromosome, which not only solved the above limitations, but also achieved stable expression of T7 RNAP.

In addition, the addition of IPTG is harmful to the cells, and the survival rate of the strain BL21 (DE3) was greatly reduced after 24 hours of fermentation, which was also verified in the above data (Fig. 3b, 3d, and 3f). It had been proved that the concentration of IPTG which was only less than 0.1mM was non-toxic to cells [25], but this concentration can not meet the requirements of our usual fermentation experiments. The three inducible promoters selected in this article, the inducers such as L-arabinose [9, 26], L-rhamnose [15, 27], anhydrotetracycline [28] had all been studied to control gene expression. Chou et al. developed the L-arabinose-induced pET system in the strain JM109 (DE3), and the production of penicillin acylase (PAC) was significantly improved, which proved that arabinose was more effective as an inducer than IPTG [29]. There was also study to prove that induction with L-arabinose can serve as a substitution to combined induction with 1 mM IPTG [9]. mLacI was placed under the P_{rhaBAD}, which can strictly control the target gene and do not affect the growth of the strain, and L-rhamnose-inducible promoter had been widely used in the production of membrane proteins and secreted proteins of *E. coli* [27]. Recently, a new gene regulation tool based on anhydrotetracycline-inducible promoter had been developed [30]. Anyhow, a large number of studies had proved that the inducer used in this paper is non-toxic or slightly toxic to cells. Therefore, the promoter engineering in this study can alleviate the pressure of cell survival caused by external environment and reduce the economic cost of protein production.

Conclusion

Tunable protein expression is crucial for synthetic and system biology. However, the BL21 (DE3) is not suitable for the expression of all proteins, such as membrane proteins and autolysable proteins. In this work, three T7 RNAP expression engineered *E. coli* regulated by inducible promoters were constructed, and were further used for the production of GDH and membrane proteins. Compared with P_{LacUV5}, the basal leakage expression of the three inducible promoters was lower, which can accurately regulate the expression of T7 RNAP. The strain BL21 (DE3) had the best production effect before 18h. However, the fluorescence intensity of the three engineered strains were all higher than BL21 (DE3) after 24h of fermentation, and the average ratio of plasmid-carrying cells were about 98%. The expression of GDH and membrane proteins were further optimized by prolonging the fermentation time without affecting the survival rate of the engineered strains. We developed three robust and novel engineered strains, which provided more host choices for recombinant proteins production.

Declarations

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Competing interests

The authors declare that they have no competing interests.

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Figures

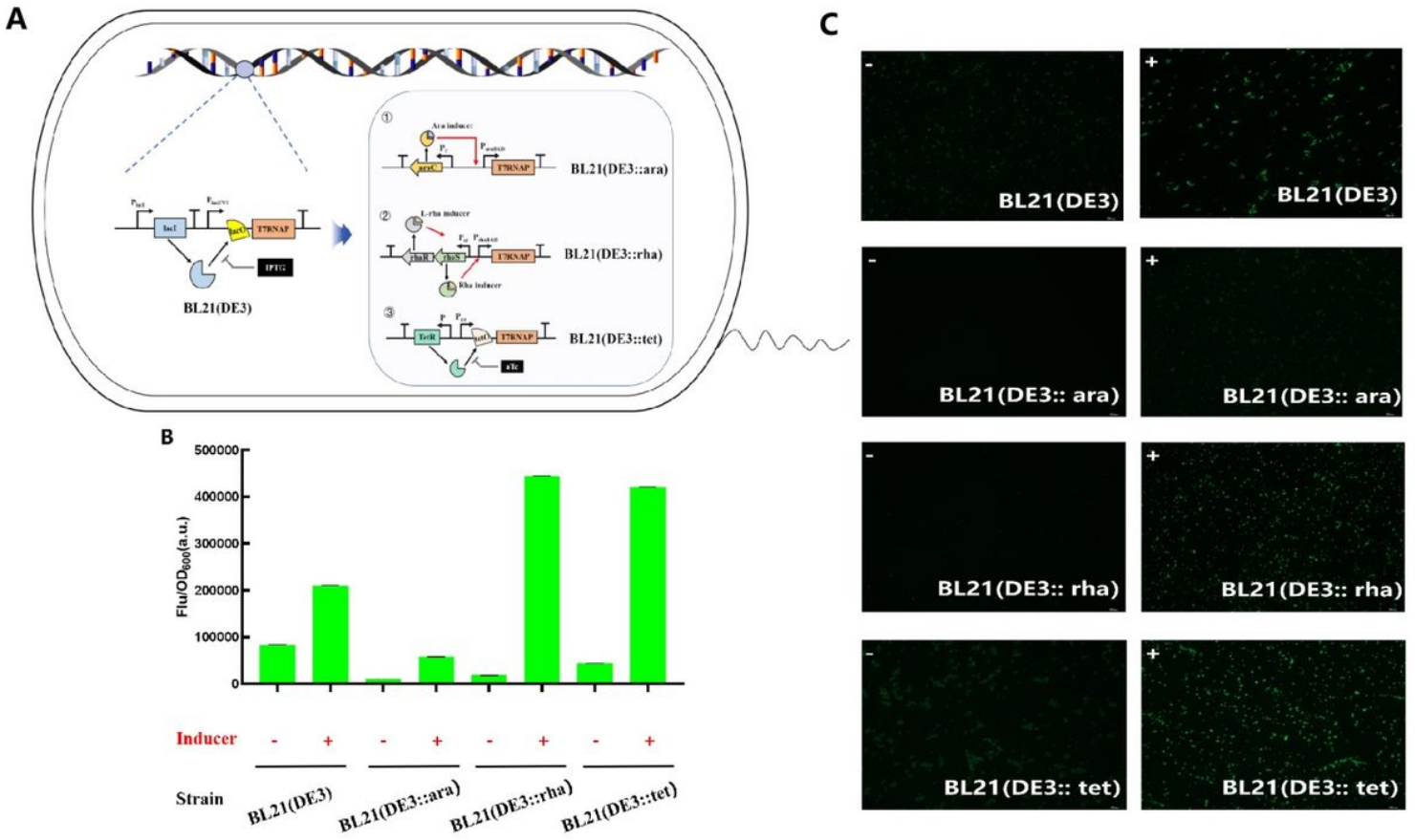

Figure 1

Construction of three engineering strains and fluorescence intensity of EGFP fermentation for 18h. (A) Genetic design of engineered T7RNAP in E. coli strains BL21 (DE3). Three engineered T7RNAP expression strains which containing different inducible promoters were constructed. (B) Fluorescence intensity of different strains containing EGFP plasmid with or without inducer and Fluorescence diagram (C). The error bar denotes the standard deviation of the mean from the three replicates.

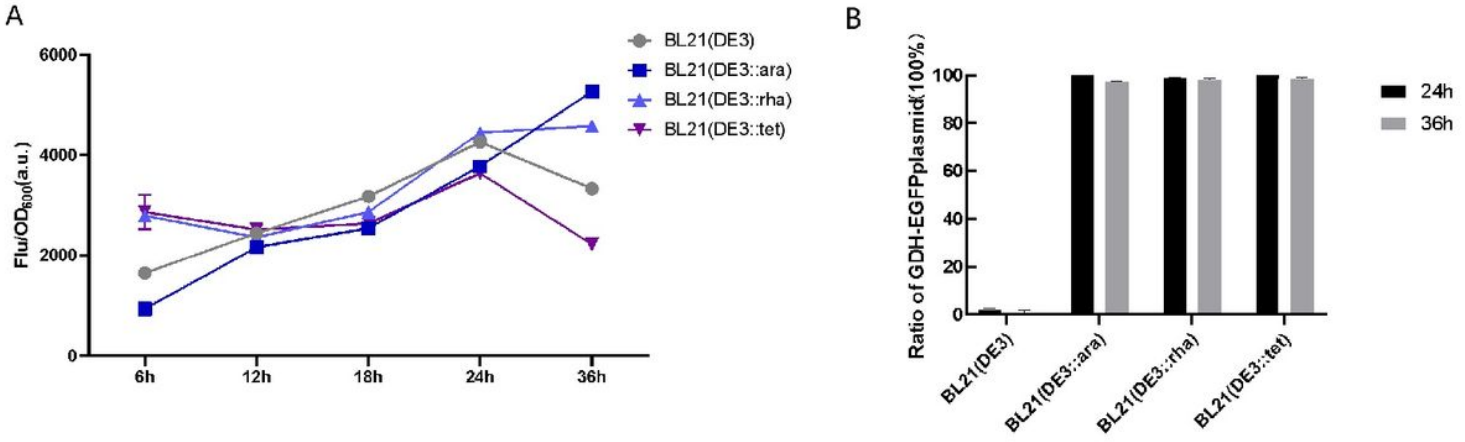

Figure 2

Expression of GDH in different strains. (A) Fluorescence intensity of GDH in the whole fermentation process. (B) The percentage of plasmid-carrying cells of BL21 (DE3), BL21 (DE3::ara), BL21 (DE3::rha) and BL21 (DE3::tet) was tested during the entire fermentation period. Values and error bars represent the means and the deviations from triplicate experiments. GDH, glucose dehydrogenase.

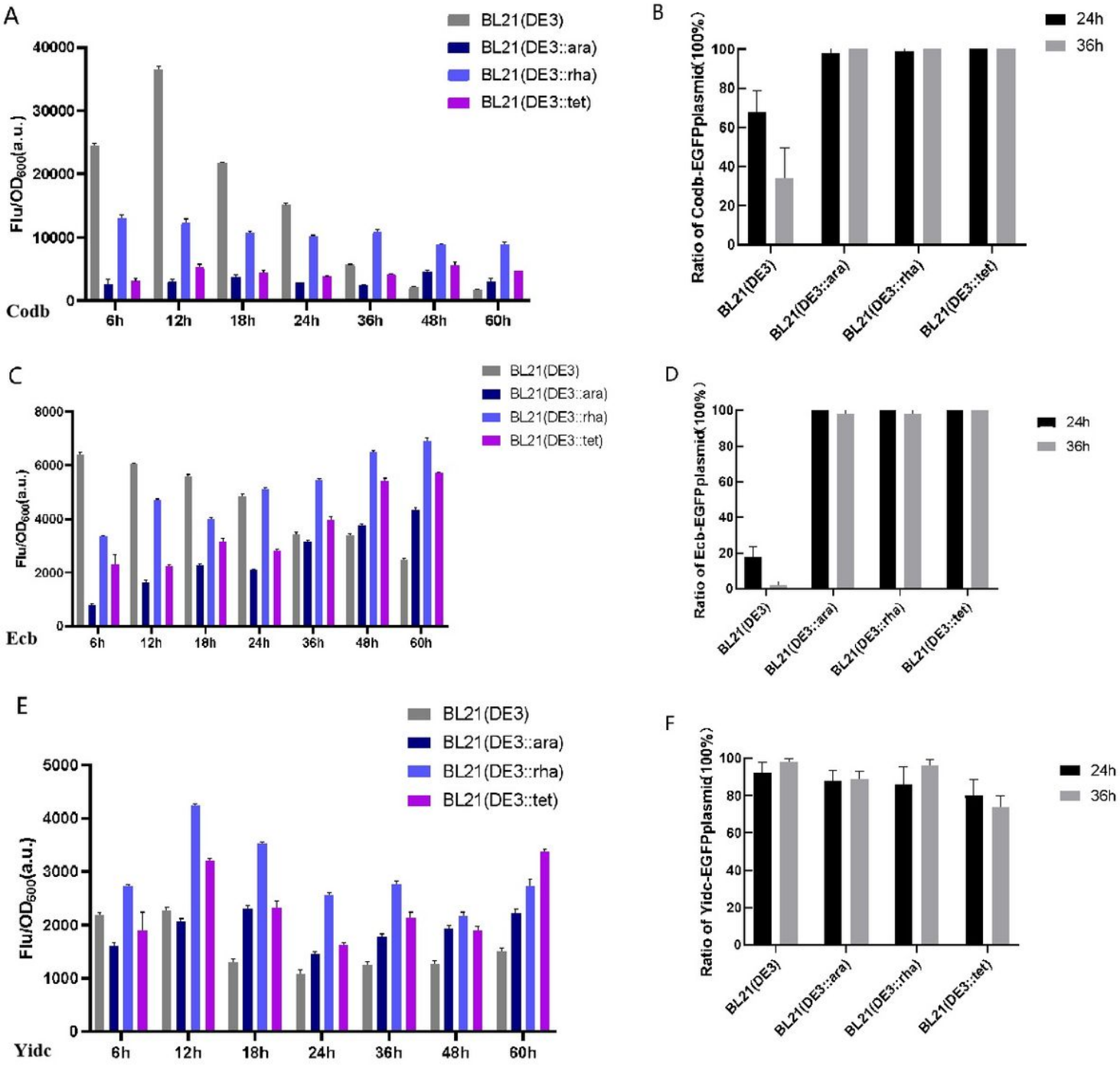

Figure 3

Expression of three membrane proteins in three engineering strains and control BL21 (DE3). (A) Fluorescence intensity of Codb of BL21 (DE3), BL21 (DE3::ara), BL21 (DE3::rha), BL21 (DE3::tet) in the whole fermentation process and the percentage of plasmid-carrying cells in (B). (C) Fluorescence

intensity of Ecb of BL21 (DE3), BL21 (DE3::ara), BL21 (DE3::rha), BL21 (DE3::tet) in the whole fermentation process and the percentage of plasmid-carrying cells in (D). (E) Fluorescence intensity of Yidc of BL21 (DE3), BL21 (DE3::ara), BL21 (DE3::rha), BL21 (DE3::tet) in the whole fermentation process and the percentage of plasmid-carrying cells in (F). Values and error bars represent the means and the deviations from triplicate experiments. CodB, E. coli cytosine transporter protein. Ecb, E. coli F-ATPase subunit b. YidC, E. coli membrane protein insertase/foldase.

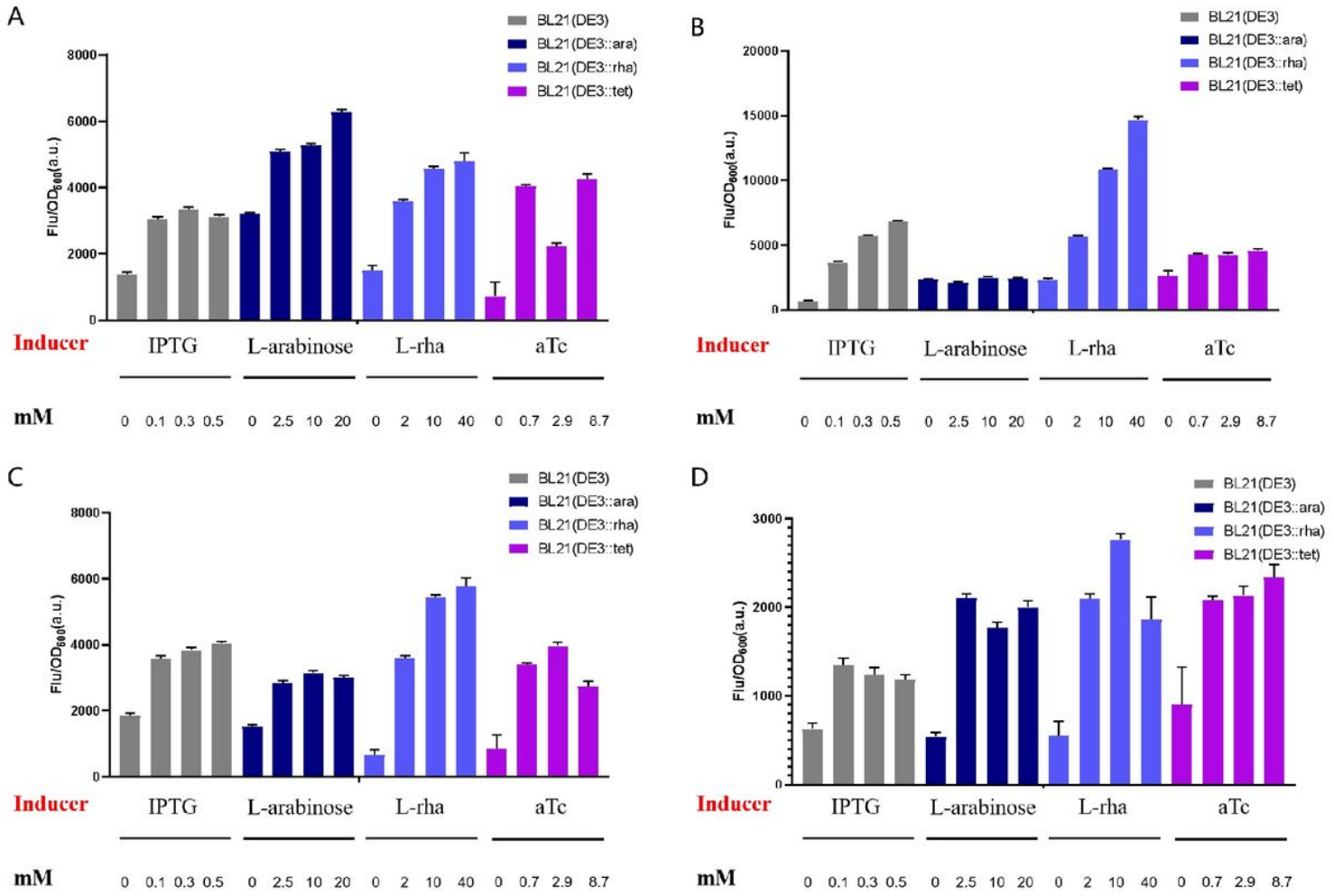

Figure 4

Tunable expression of GDH and three membrane proteins in three engineering strains and control BL21 (DE3) under different inducer concentrations in 36h. (A) Tunable expression of GDH. (B) Tunable expression of Codb. (C) Tunable expression of Ecb. (D) Tunable expression of Yidc. Values and error bars represent the means and the deviations from triplicate experiments.

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