A novel gene expression system for *Ralstonia eutropha* based on the T7 promoter

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Abstract

Background: *Ralstonia eutropha* (syn. *Cupriavidus necator*) is a model microorganism for the metabolism of polyhydroxyalkanoates (PHAs) and a potential chassis for protein expression. Although current plasmid systems for *R. eutropha* provide a basic platform for gene expression, the performance of the induction systems is still limited. In addition, the sizes of the cloned genes is limited due to the large sizes of the plasmid backbones.

Results: In this study, an *R. eutropha* T7 expression system was established by integrating a T7 RNA polymerase gene driven by the P_{BAD} promoter into genome of *R. eutropha*, and cloning the T7 promoter into a pBBR1-derived plasmid for gene expression. In addition, the essential sequence necessary for pBBR1 plasmid replication was identified, and the redundant parts were deleted, reducing the expression plasmid size to 3392 bp, and the electroporation efficiency was improved 4 times. As a result, the highest expression level of Rfp was enhanced slightly, and the L-arabinose concentration necessary for induction was decreased 20 times.

Conclusions: *R. eutropha* with the T7 expression system provides an efficient platform for protein expression and synthetic biology applications.

Background

Ralstonia eutropha, also known as *Cupriavidus necator*, is a facultative chemolithoautotrophic bacterium which fixes CO₂ via the Calvin-Benson-Bassham (CBB) cycle[1]. It is a model microorganism for studying the metabolism of polyhydroxyalkanoates (PHAs), which it can accumulate to large amounts under certain conditions[1, 2]. When phosphorus was limited, the weight of poly(3-hydroxybutyrate) (PHB) accumulated in the cells exceeded 80% of the cell dry weight[3], which makes *R. eutropha* a potential industrial strain for PHA production.

Metabolic engineering and synthetic biology were used to engineer *R. eutropha* for the production of biofuels and other valued-added products, including isobutanol[4], 3-methyl-1-butanol[4], methyl ketones[5], hydrocarbons[6], ethanol[7], isopropanol[8, 9], fatty acids[10] and α -humulene[11]. It is also a potential chassis for the production of industrial enzymes due to its ability to suppress the formation of inclusion bodies[12], lack of acidic byproducts, and high obtainable cell density[3].

A robust gene expression toolbox which includes suitable host strains, plasmid vectors and promoters is essential for metabolic engineering and synthetic biology. Plasmids derived from pBBR1, IncP and IncQ can replicate in *R. eutropha* and were used as plasmid vectors[6]. Promoters used in *E. coli*, such as Plac and Ptac[13], were found to be expressed constitutively in *R. eutropha*, while native promoters from *R. eutropha*, such as PphaC, PphaP, Pphb, PacoE, PacoD and PpdhE, were also evaluated[13, 14]. In addition, promoters induced by L-arabinose[6], L-rhamnose[15], and anhydrotetracycline[16] were used in *R. eutropha*. However, most of the plasmid vectors and promoters mentioned above were not used widely. Although broad-host-range plasmid vectors derived from pBBR1 with the L-arabinose induced promoter

P_{BAD} were used widely in *R. eutropha*, the vectors were large, which limited gene cloning and plasmid transformation.

When the enzyme organophosphohydrolase (OPH) was expressed in engineered *R. eutropha*, its content reached more than 10 g/L, which was a more than 100-fold increase compared to OPH expression in *E. coli* [12]. This indicated that *R. eutropha* is a potential industrial protein expression host. In this study, a gene expression system based on the T7 promoter was constructed to improve the current gene expression technology for *R. eutropha* and make it more widely available as a host.

Materials And Methods

Strains and culture conditions

The strains used in this study are listed in Table S1. *E. coli* was cultured at 37°C in lysogeny broth medium (LB, 10 g/L peptone, 5 g/L yeast extract, 10 g/L NaCl). Streptomycin (100 µg/mL) or kanamycin (50 µg/mL) was added to the medium where appropriate. *R. eutropha* was cultured in LB at 30°C. Gentamycin (10 µg/mL) or kanamycin (200 µg/mL) was added where appropriate.

Primers and Plasmids

Primers (Table S2) used for the construction of plasmids (Table S3) in this study were designed using j5 DeviceEditor[17]. The other primers were designed using Clonemanager software. High fidelity DNA polymerase was purchased from Takara (Dalian, China) or New England Biolabs (USA). BsaI restriction endonuclease and T4 ligase were purchased from New England Biolabs (USA) and Thermo-Fisher Scientific (USA), respectively. Plasmids were constructed using Golden Gate[18], Gibson[19] or CPEC[20] methods, and used to transform *E. coli* or *R. eutropha*. Plasmids were extracted using the AxyPrep Plasmid Miniprep Kit (AXYGEN, China) according to the manufacturer's instructions, with minor modifications.

Assessment of RFP expression levels

Red fluorescence intensity was measured using a microplate reader (Infinite M200 PRO, TECAN) after the strains were induced with L-arabinose for 48 hours. The excitation and emission wavelengths were 585 and 620 nm respectively[6]. Optical density at 600 nm (OD₆₀₀) was also measured using the microplate reader. The RFP expression levels were determined as the ratio of the red fluorescence intensity to the OD₆₀₀.

Identification of the region necessary for plasmid replication

Different primers for Golden Gate were designed using j5 DeviceEditor, and PCR was implemented using the plasmid pBBR1-P_{T7}-rfp as template. The products of the PCR were purified using the SanPrep DNA Purification Kit (Sangon Biotech, China), and then cyclized via Golden Gate assembly. As a result, some

regions of the pBBR1-P_{T7}-rfp were deleted, and the minimized plasmids were used to transform *E. coli*. Transformed colonies would be obtained if the core region for plasmid replication was intact, while transformation would be unsuccessful if the core region were damaged.

Electroporation of *R. eutropha* and evaluation of transformation efficiency

Competent cells of *R. eutropha* were prepared according to the protocol described in our previous work[21]. Briefly, *R. eutropha* was cultured in 100 mL LB at 30°C and 200 rpm, then chilled on ice for 5-10 min after the OD₆₀₀ reached 0.8. The cells were collected by centrifugation at 3000× g for 5 min and washed with ice-cold sterile 10% (V/V) glycerol three times. Finally, the cells were suspended in about 0.5 mL 10% glycerol and 0.1 mL portions transferred to sterile 1.5-mL tubes. The prepared *R. eutropha* competent cells were used immediately, or frozen in liquid nitrogen and preserved at -80°C for a few weeks.

Electroporation of *R. eutropha* was implemented according to the protocol described in our previous work[21]. Briefly, appropriate plasmids were added to the competent cells and mixed gently. The mixture was transferred into a pre-chilled 2-mm electroporation cuvette (Bio-Rad, USA), and incubated on ice for 5 min, after which electroporation was performed at a voltage of 2.3 kV. Immediately afterwards, 1 mL of LB preincubated at 30°C was added to the cells. All the cells were transferred to a sterile 1.5 mL tube and incubated at 30°C for 2 h. Then, a portion of the cells was spread on LB agar plates with appropriate antibiotics, and cultured at 30°C until colonies were visible. Electroporation efficiencies were calculated as the ratio of colony-forming units (CFU) to the amount of the used plasmid DNA.

Results And Discussion

Construction of a T7 gene expression system for *R. eutropha*

The T7 RNA polymerase gene was cloned from *E. coli* BL21(DE3), modified to be driven by the L-arabinose induced promoter P_{BAD}, and the *rfp* gene was cloned under the control of a T7 promoter (taatacgactcactataggg). The resulting plasmid pj5_00019, which was based on pBBR1, was introduced into either *E. coli* S17-1[22] or *R. eutropha* C5[21]. However, we found that *rfp* was expressed in *E. coli* S17-1, but was not expressed in *R. eutropha* C5 with or without L-arabinose induction. We considered that the failure of expression in *R. eutropha* might due to the multiple copies of the T7 RNA polymerase genes expressed from the multicopy plasmid.

To verify the hypothesis, *R. eutropha* strain C5T7 was constructed by integrating the RNA polymerase gene with the P_{BAD} promoter and *araC* into the *H16_A0666* locus on the *R. eutropha* C5 chromosome. The expression plasmid pBBR1-P_{T7}-rfp was constructed by cloning the T7-promoter-driven *rfp* into the pBBR1 backbone. After electroporation into *R. eutropha* C5T7, the strain C5T7(pBBR1-P_{T7}-rfp) showed visible red color when induced (Fig. 1).

Minimization of the expression plasmid vector pBBR1-P_{T7}-rfp

The pBBR1-P_{T7}-rfp plasmid expressed in *R. eutropha* C5T7 had a size of 4751 bp, which limited the size of genes that can be cloned into the vector. Considering the origin of the plasmid, there might be redundant regions that are non-essential for plasmid replication and could be deleted to minimize the plasmid. The DNA cassette acttaaaaatcaacaacttaaaaa is A+T rich and contains two direct repeats, and was assumed to be the replication origin of pBBR1-derived plasmids [23]. Thus, adjacent regions of the putative replication origin were gradually deleted via PCR followed by Golden Gate cyclization. As a result, a series of circular DNA cassettes were constructed, among which only pj5_00030 could be introduced into *E. coli* successfully, which confirmed that only this plasmid contained a functional minimized origin of replication (Fig 2a). The putative replication origin with about 400 bp of its upstream sequence was conserved in pj5_00030 and was essential for plasmid replication. The minimized plasmid vector pj5_00030 was designated as pBBR1-P_{T7}-rfp(mini) (Fig 2b).

Gene expression efficiency of the *R. eutropha* T7 expression system

To determine the performance of the *R. eutropha* T7 expression system with the minimized expression plasmid, pBBR1-P_{T7}-rfp(mini) was transferred into *R. eutropha* C5T7 via electroporation for expression analysis. Rfp expression was induced by different concentrations of L-arabinose. The results showed that the expression level of Rfp increased with the increase of L-arabinose concentration in the range from 0 to 0.1 mg/mL L-arabinose. When 0.1 mg/mL L-arabinose was added for induction, the expression level of RFP was highest but decreased when L-arabinose exceeded that threshold (Fig 3a). Compared with pBBR1-P_{BAD}-rfp in *R. eutropha* H16, the highest RFPP expression level of *R. eutropha* C5T7(pBBR1-P_{T7}-rfp(mini)) was slightly improved. Especially, due to the higher sensitivity of the T7 expression system, the L-arabinose concentration needed for the highest induction level was lowered 20 times compared with the traditional P_{BAD} system (Fig 3a), which can bring significant cost savings in industry.

Electroporation efficiency of the plasmid

To evaluate the electroporation efficiency of the pBBR1-derived plasmids, pBBR1-P_{BAD}-rfp, pBBR1-P_{T7}-rfp and pBBR1-P_{T7}-rfp(mini) were extracted from *E. coli* S17-1, and then individually electroporated into *R. eutropha* C5T7 competent cells. The results showed that the electroporation efficiency of pBBR1-P_{T7}-rfp(mini) was significantly higher than that of pBBR1-P_{BAD}-rfp or pBBR1-P_{T7}-rfp, probably due to the reduced plasmid size (Fig 3b). Thus, the minimized vector could provide a better cloning efficiency.

Conclusions

In this study, an optimized T7 expression system for *R. eutropha* was constructed for protein expression, synthetic biology manipulation or other applications. The system has a few advantages over previous ones. First, with the high sensitivity of the T7 system, the highest expression level was slightly enhanced, and the L-arabinose concentration for induction was decreased 20 times, offering great cost saving. Secondly, due to the minimized size of the expression vector, the electroporation efficiency was improved 4 times ($p < 0.05$), which also makes the plasmid a better vector for accommodating large DNA cassettes.

Declarations

Ethical Approval and Consent to participate

Not applicable

Consent for publication

I hereby give the journal of Microbial Cell Factories the right and permission to publish this article.

Availability of data and material

We provide supporting and necessary data for publication of the article. All supporting data is present in the article and the supplemental material documents.

Authors' contributions

XB, HM, LZ, LL and LS planned and performed the experiments, analyzed and interpreted the data. ZC, BC and ZX supervised the study, designed the experiments and analyzed and interpreted the results. XB and HM wrote the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Supplementary Information

Additional file 1: Table S1, Table S2 and Table S3

Additional file 2: Sequence of pBBR1-P_{T7}-rfp (mini)

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Figures

Figure 1

Gene arrangement of integration locus of *R. eutropha* expression host and plasmid vector. (a) Schematic illustration of the gene arrangement of integration locus of *R. eutropha* C5T7. (b) Profile of gene expression vector pBBR1-PT7-rfp.

Figure 2

Minimized plasmid vector pBBR1-PT7-rfp(mini). (a) PCR verification of circular DNA. (b) Profile of plasmid pBBR1-PT7-rfp(mini). F: the primer pBBR1_2303F, R: the primer pBBR1_2303R.

Figure 3

The performance of pBBR1-PT7-rfp(mini), including expression strength and electroporation efficiency. (a) Red fluorescent intensity of *R. eutropha* C5T7(pBBR1-PT7-rfp(mini)) with induction of different concentrations of L-arabinose. *: Rfp intensity of pBBR1-PBAD-rfp in *R. eutropha* H16, 2000 μg/mL L-arabinose was added for induction. (b) electroporation efficiency of pBBR1-pBAD-rfp, pBBR1-PT7-rfp and pBBR1-PT7-rfp(mini) into C5T7 strain. The results represent three biological replicates. *: difference was significant ($P < 0.05$).

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