Engineered chromosome-based T7 RNA polymerase in *Escherichia coli* W3110 for orthogonal T7 promoter circuit as a cell factory

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Research

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Abstract

Background

Orthogonal T7 RNA polymerase (T7RNAP) and T7 promoter were powerful tools to mediate the protein expression. Moreover, *Escherichia coli* W3110 strain possesses more advantages than the B strain due to more heat shock proteins and higher tolerance to chemicals. Therefore, implementation of T7-based system in W3110 strain is a conceivable strategy to develop the cell factory.

Results

Three novel W3110 strains with chromosome-equipped T7RNAP (i.e W3110:IL5, W3110::L5 and W3110::pI) were engineered to demonstrate the feasibility on protein expression and chemical production. At first, the LacZ and T7RNAP with IPTG induction showed higher expression levels in W3110 derivatives than that in BL21(DE3). The plasmids with and without *lacI*/*lacO* repression were used to investigate the protein expression of super-fold green fluorescence protein (sfGFP), Cas9, carbonic anhydrase (CA) and lysine decarboxylase (CadA). All the proteins were expressed higher and enzymatic functions were better in W3110::L5 and W3110::pI. Moreover, the highest cadaverine production, lysine consumption and the yield were obtained in W3110::L5(+) strain with pET28a(+)-CadA which reached 32.2 g/L, 45 g/L and 91.7% at 24 h, while the W3110::pI(-) strain with pSU-T7-CadA achieved 36.9 g/L, 43.8 g/L and 103.4% at 12 h which is unnecessary of inducer.

Conclusion

Inducer and *lacI*/*lacO* regulators on chromosome and plasmid have been investigated in W3110 strains with T7RNAP. The newly engineered W3110::L5 and W3110:pI both possessed similar protein expression compared to commercial BL21(DE3). Furthermore, among all strains, W3110::pI displayed the greatest potential as cell factory in the future.

Introduction

It is indispensable to develop the recombinant technology which enables enhanced enzyme expression to endorse high value chemicals production in recent years. The recombinant technology inspired the scientific studies of enzymes, protein and protein interactions and the *in vivo* chemical production for industrial applications [1–3]. In addition, pharmaceutical and biotechnology companies applied recombinant technology to produce miscellaneous therapeutic proteins with the advantages of high output of the desired proteins and reduced cost due to the simplified purification processes. For example, recombinant insulin was the first practical therapeutic protein produced by *E. coli* and approved by the Food and Drug Administration [4]. Moreover, approximately 30% recombinant proteins without

glycosylation are produced from *E. coli* [5]. With different demands, more prokaryotic and eukaryotic chassis have been explored to produce recombinant therapeutic proteins.

Actually, *E. coli* has been widely used as a genetic strain due to the high growth rate, ease in culturing, well-characterized genome, and high availability of versatile genetic tools [6]. With the development of genetic tools and bioinformatics, it has been shown more possibilities to express foreign pathways and to produce valuable chemicals via manipulating the metabolic pathways in *E. coli* [7–9]. The cellular engineering in *E. coli* was originated from plasmid construction [10], followed by pET system for over-expression under orthogonal T7RNAP and promoter [11], homologous recombination [12], and also CRISPR/Cas9 technology for genome editing recently [13–16].

The *E. coli* B strain and derivatives lacking Lon and OmpT protease empowers producing heterologous proteins without protease attack. One such derivative, BL21(DE3), infected by λDE3 lysogen and exposed to the *T7 gene 1*, possesses T7RNAP on the chromosome controlled by the P_{LacUV5} promoter and the nearby *lac*I/*lac*O orthogonal regulator [11]. P_{LacUV5} is a mutant promoter of the native *lac* promoter, with a low sensitivity to glucose [17]. The expression is regulated by the *lac*I repressor, which binds to the *lac* operator (*lac*O) in the absence of lactose. The repression can be removed by adding an inducer such as lactose or isopropyl-β-D-thiogalactopyranoside (IPTG) [18], resulting in target gene expression by the orthogonal T7 promoter. IPTG is frequently added to the T7 system due to its stability and effectiveness [19]; however, IPTG is expensive and uneconomic for industrial applications of low value-added products [20].

Alternatively, the K-12 strains such as MG1655 and W3110, are commonly used as they express more heat shock genes, are less sensitive to certain stress and also possess higher rates of glucose consumption [21–22]. A comparative proteomics study between BL21 and W3110 manifested that W3110 maintained growth and metabolism at lower oxygen levels, thus enabling foreign protein to be gradually expressed [23]. Therefore, W3110 strain has also been applied to produce various chemicals, including L-methionine [24–25], L-homoserine [26], and L-malate [27].

In this study, a powerful T7 system was equipped in W3110 strain to establish an alternative cell factory as a protein expression platform and microbial chemical production. First, T7RNAPs using different promoters, i.e., P_{LacUV5} promoter with or without additional LacI repressor and P_{LacI} promoter, were inserted to the W3110 chromosome by a conditional replication, integration, and modular (CRIM) system [28] to develop three engineered W3110 strains. Then, the effect of *lac*I/*lac*O was explored by sfGFP as a proof-of-concept. To be a powerful cell factory, new engineered strains were applied to express Cas9 for gene editing, carbonic anhydrase (CA) for capture of carbon dioxide and lysine decarboxylase CadA for production of cadaverine (DAP) as a precursor of bio-nylon materials. We attempted to explore the brighter opportunity of W3110 strain as a new platform to express heterologous protein and produce chemicals.

Materials And Methods

Bacterial strains and culture conditions

The bacterial strains used in this study are listed in Table 1. The *E. coli* DH5α was used for plasmid construction, and BL21(DE3) was applied for gene expression, while W3110 was engineered to equip with a chromosome-based T7RNAP by HK022 phage attack site. All *E. coli* strains were cultivated in Luria–Bertani (LB) medium at 37 °C with constant shaking at 200 rpm. Antibiotics were added to the following final concentrations as needed: ampicillin (100 μg/mL), kanamycin (50 μg/mL), and chloramphenicol (25 μg/mL).

Table 1
List of *E. coli* strains and plasmids used in this study

Strains and plasmids	Description	Source or Accession number
Strains		
W3110	F-, λ^-, *IN(rrnD-rrnE)1*, *rph-1*	CGSC #4474
BL21	F^- *ompT gal dcm lon* $hsdS_B(r_B^-m_B^-)$ $[malB^+]_{K-12}(\lambda^S)$	NEB #C2530H
BL21(DE3)	F^- ompT gal dcm lon $hsdS_B(r_B^-m_B^-)$ λ(DE3 [lacI lacUV5-T7p07 ind1 sam7 nin5]) $[malB^+]_{K-12}(\lambda^S)$	NEB #C2527I
W3110::IL5	F-, λ^-, *IN(rrnD-rrnE)1*, *rph-1* attB (HK022)::lacI- P_{LacUV5}- lacZ'- *T7 gene 1*	This study
W3110::L5	F-, λ^-, *IN(rrnD-rrnE)1*, *rph-1* attB (HK022):: P_{LacUV5}- lacZ'- *T7 gene 1*	This study
W3110::pI	F-, λ^-, *IN(rrnD-rrnE)1*, *rph-1* attB (HK022):: P_{LacI}- *T7 gene 1*	This study
Plasmids		
pAH69	4951 bp, Amp^R, Rep101 ori, repA101(ts), HK022 integrase, cIts857 protein	[28]
pHK-Km	2539 bp, Kan^R, R6K ori, FRT- HK022 attp- FRT	[29]
pDS3.0- HsdNC-T7RNAP	11966 bp, Amp^R, Gm^R, R6K ori, Hsd-N, P_{LacI} promoter, *T7 gene1*, Hsd-R, *sac*B	ACY75835
pHK-IL5-Km	6933 bp, Kan^R, R6K ori, FRT- HK022 attp- lacI-P_{Lacuv5}- T7 promoter- lacZ'- *T7 gene1*- FRT	This study
pHK-L5-Km	5766 bp, Kan^R, R6K ori, FRT- HK022 attp- P_{Lacuv5}- T7 promoter- lacZ'- *T7 gene1*- FRT	This study
pHK-pI-Km	6586 bp, Kan^R, R6K ori, FRT- HK022 attp- Hsd-N- P_{LacI} promoter- *T7 gene1*- Hsd-R- FRT	This study
pET28a (+)-sfGFP	6013 bp, Kan^R, pBR322 ori, *lacI*, T7 promoter, *lacO*, *sfGFP*	This study
pSU-T7-sfGFP	9471 bp, Cm^R, pUC ori, T7 promoter, *sfGFP*	[35]
pSU-PlacI-sfGFP	3099 bp, Cm^R, pUC ori, PlacI promoter, *sfGFP*	[41]

Strains and plasmids	Description	Source or Accession number
pSU-PlacUV5-sfGFP	3099 bp, Cm^R, pUC ori, PlacUV5 promoter, *sfGFP*	This study
pSU- PlacUV5-NRBS-sfGFP	3099 bp, Cm^R, pUC ori, PlacUV5 promoter, native RBS, *sfGFP*	This study
pSU-PlacUV5-LacZα'-sfGFP	3571 bp, Cm^R, pUC ori, PlacUV5 promoter, LacZα', *sfGFP*	This study
pET32a-SyCA	6595 bp, Amp^R, pBR322 ori, *trx*A, *lacI*, T7 promoter, *lacO*, *SyCA*	[32]
pET21a (+)-Cas9	9471 bp, Amp^R, pBR322 ori, *lacI*, T7 promoter, *lacO*, *SpCas9-6His*	Lab stock
pET20b (+)-Cas9	7694 bp, Amp^R, pBR322 ori, ROP, T7 promoter, *Spcas9-6 His*	This study
pET28a (+)-CadA	7498 bp, Kan^R, pBR322 ori, *lacI*, T7 promoter, *lacO*, *cad*A	This study
pSU-T7-CadA	4255 bp, Cm^R, pUC ori, T7 promoter, c*ad*A	This study

Construction Of CRIM Plasmids With T7RNAP Under Different Promoters

The pHK plasmid was used for inserting an integrated fragment including the HK022 *att*P, FRT, and multiple cloning sites [29]. The first CRIM plasmid, pHK-IL5T7-Km, was designed to create a strain that imitates BL21(DE3), constructed by polymerase chain reaction (PCR) of the BL21(DE3) genome with primers *Eco*RI-lacI-F and *Pst*I-T7P-R and digested with *Eco*RI and *Pst*I, cloned into the pHK-Km backbone. The pHK-L5T7-Km was constructed by PCR from pHK-IL5T7-Km with primers *Eco*RI-P_{lacUV5}-F and *Pst*I-T7P-R, while plasmid of pHK-pIT7-Km was cloned by PCR from pDS3.0-HsdNC-T7RNAP with primers *Eco*RI-PlacI-T7P-F and *Pst*I-PlacI-T7P-R to obtain T7RNAP under different promoters. The primers used are listed in Table S1.

Engineered Chromosome-based T7RNAP In W3110

Site-specific recombination was applied to integrate T7RNAP into the W3110 genome. This process requires the CRIM plasmid as mentioned above, which includes an *att*P phage attachment site complementary to the *att*B phage attachment site on the genome, and a helper plasmid that includes integrase [28]. The helper plasmid pAH69 was transformed into the host strain at first. To prepare competent cells, a transformant cultivated at 30 °C until the OD_{600} reached 0.3; the temperature then

increased to 39 °C for 30 min to induce integrase production. The heat-shock transformation was used to transfer the CRIM plasmid into the host strain, followed by recovery at 37 °C for 2–3 h. Afterwards, positive colonies were screened on LB agar medium with 17 μg/mL kanamycin at 37 °C to check the removal of the pAH69. To construct marker-free strains, pCP20 plasmid was further transformed to remove the resistance fragment flanked by FRT sites by FLP recombinase. One colony was randomly selected and pre-cultured in LB with antibiotic at 30 °C. The cells were inoculated in LB broth without antibiotic and cultured at 37 °C for 6–8 h, followed by transferring to 39 °C for 1 h. The cells were then diluted and spread on an LB plate at 37 °C. After overnight cultivating, the colonies were screened on selective plates.

Construction Of Expression Vectors

The pET28a(+)-sfGFP plasmid was constructed by amplifying the sfGFP fragment from pSU-placI-sfGFP with primers *Nde*I-sfGFP-F and *Xho*I-sfGFP-R, followed by digestion with *Nde*I and *Xho*I and cloning into pET28a(+)-RFP, which was digested with the same digestion enzymes in advance. The pET20b(+)-Cas9 was constructed by digestion with *Xba*I and *Xho*I from pET21a(+)-Cas9 to obtain the *cas9* fragment. The fragment was cloned into pET20b(+), which was digested by *Xba*I and *Xho*I in advance. The *cad*A fragment was amplified with *Hind*III-CadA-F and *Bgl*II-CadA-R from *E. coli* MG1655, digested with *Hin*dII and *Bgl*II, and inserted into the pSU-T7 backbone, creating the pSU-T7-CadA plasmid. The pSU-P_{LacUV5}-sfGFP was constructed by replaced the promoter of pSU-T7-sfGFP and the fragment of P_{LacUV5} was amplified from pHK-L5-Km with primers *Xba*I-L5-F and *Bam*HI-L5-R. The PCR fragment was digested with *Xba*I and *Bam*HI and cloned into pSU-T7-sfGFP. The pSU-P_{LacUV5}-NRBS-sfGFP was constructed by replaced the promoter and RBS of pSU-T7-sfGFP. The inserted fragment was amplified with primers *Xba*I-L5-F and *Hin*dIII-L5-R from pHK-L5-Km, further digested with *Xba*I and *Hin*dIII and cloned into pSU-T7-sfGFP. The pSU-P_{LacUV5}-LacZα'-sfGFP was constructed by inserted the fragment of P_{LacUV5}-LacZα' into the backbone of pSU-T7-sfGFP. The inserted fragment was amplified with *Xba*I-L5-F and *Hin*dIII-L5Z-R from pHK-L5-Km. All the plasmids are shown in Table 1.

Culture And Induction

For sfGFP protein and fluorescence analysis, the samples were inoculated with 2% (*v/v*) pre-culture broth in 10-mL glass cultivation tubes and cultivated in a 37 °C incubator. IPTG was added to a final concentration of 0.1 mM. For protein analysis of Cas9, the samples were inoculated with 2% (*v/v*) pre-culture broth in a 100-mL flask with 10 mL LB broth, and the desired antibiotic was added. When the cell density achieved an OD_{600} of 0.5 to 0.8, IPTG was added, and transfer to cultivate in a 22^{o}C incubator for 12 h. The cultivation for the activity measurement of SyCA, 20 mL LB broth was inoculated with 2% (*v/v*) pre-culture broth in a 100-mL flask. As OD_{600} reached 0.6 to 0.8, IPTG was added at three different concentrations of 0.005, 0.01 and 0.1 mM, and 0.5 mM zinc ions (supplied by $ZnSO_4$) were also added. Further keep cultivating at 37^{o}C until 12 h. For the protein analysis of CadA, 100-mL flask with 10 mL LB

broth was inoculated with 2% (*v/v*) pre-culture cell, when OD_{600} reached 0.6 to 0.8, IPTG was added from 0, 10^{-5}, 10^{-4}, 10^{-2}, 10^{-3} to 0.1 mM for pET28a(+)-CadA and 0.1 mM for pSU-T7-CadA.

Measurement Of Cell Growth And Fluorescence Intensity

The cell growth was monitored by absorbance at 600 nm (OD_{600}) and the fluorescence intensity was detected by a SpectraMax M2 microreader (Molecular Device, USA) with excitation at 480 nm and emission at 510 nm. All experiments were performed in triplicates.

Protein Expression, Quantification And Identification Analysis

The recombinant protein expression was analyzed by 8%, 10% or 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After a 12-h culture, the cells were harvested by centrifugation (12000 × *g*, 3 min). The cell concentration was adjusted to OD 4, and the cells were suspended in dH_2O. The supernatant and cell debris were separated by centrifugation after the whole cells were degraded by a One Shot instrument (Constant Systems, UK). 1D electrophoretic protein separation was achieved by following a standard SDS-PAGE protocol (80 V, 30 min; 120 V, 70 min). Afterwards, the gel was stained with Coomassie brilliant blue G-250 dye for visualization. ImageLab® (Bio-rad) adopted from a Gaussian model was used in finding differentially expressed proteins of SDS-PAGE [30]. The target protein was sent for LC-Q-TOF mass spectrometer (Applied Biosystems, Lincoln, CA). Injected samples were first trapped and desalted on an LC-Packings PepMap™ C18 µ-Precolumn™ Cartridge (5 µm, 5 mm x 30 µm I.D.; Dionex, Sunnyvale, CA). Subsequently, peptides were eluted from the pre-column and separated on an analytical LC-Packings PepMap C18 column (3 µm, 15 cm x 75 µm I.D.) connected in-line to the mass spectrometer, at a flow rate of 200 nL/min using a 40-min gradient of 5 to 60% acetonitrile in 0.1% formic acid. Only significant hits from the individual MS/MS spectra were considered by mascot probability analysis.

In vitro **assay of Cas9-ribonucleoprotein (Cas9-RNP)**

Double-stranded DNA sgRNA was synthesized via overlap PCR to combine the crRNA fragments and the scaffold. The sgRNA was synthesized by in vitro transcription with double-stranded DNA sgRNA as the template, 10x transcription buffer, rNTP solution mixture, and T7 RNA polymerase. The crude sgRNA was purified by the phenol-chloroform method. Crude Cas9 protein was purified by His-trap metal-ion affinity chromatography with an AKTA™ start machine (GE Healthcare, UK). Imidazole was removed from the purified Cas9 protein by Amicon®100-kDa ultrafiltration (Merck, USA). The Cas9-RNP was formed by mixing equal molar ratios of purified Cas9 and sgRNA in 5x reaction buffer (100 mM HEPES, pH 7.5, 500 mM KCl, 25% glycerol, 5 mM DTT, 2.5 mM EDTA, 10 mM $MgCl_2$ at pH 8.0) at room temperature for 15 min. *In vitro* digestion was achieved by adding 100 to 200 ng DNA template at 37 °C for 1 h.

Determination of CA activity by Wilbur–Anderson unit (WAU)

The cell expressing the CA was collected and washed 3 times by deionized water and finally adjusted to OD 5 for further use. The CA activity was determined by Wilbur-Anderson Assay as our previous publication. Briefly, 9 mL ice-cold Tris-HCl (20 mM, pH8.3) buffer and 0.2 mL enzyme were mixed and put in a 20 mL sample bottle at 0^{o}C under stirring. Then, 6 mL ice-cold CO_2-saturated solution was added into sample bottle immediately. The time in second that pH value dropped down from 8.3 to 6.3 was recorded and the CA activity was calculated by a Wilbur–Anderson unit (WAU) per milliliter of sample. The definition for WAU is (T_0-T)/T, which T_0 and T is the time from pH 8.3 to 6.3 without and with CA.

In vivo **cadaverine production**

A 2% (*v/v*) pre-culture was inoculated in a 100-mL flask and cultivated in a 37 °C incubator. When the OD_{600} reached 0.5–0.8, 0.1 mM IPTG was added as an inducer, with 0.35 M lysine and 0.01 mM pyridoxal phosphate (PLP) as the substrate and co-factor, respectively. The growth, lysine conversion, and cadaverine production were measured every 6 h.

Derivatization And High-performance Liquid Chromatography (HPLC)

Lysine and cadaverine derivatives were obtained by combining 340 µL 50 mM borate buffer (pH 9), 240 µL 100% methanol, 12 µL 200 mM diethyl ethoxymethylenemalonate (DEEMM) without pretreatment, and 6 µL of the sample (< 0.1 M). The mixture was heated in a 70 °C water bath for 2 h to allow complete degradation of excess DEEMM and derivatization [31]. After derivatization with DEEMM, HPLC was performed with a binary pump, in-line degasser, auto-sampler, and column thermostat. Chromatographic separation was conducted by reverse-phase chromatography on a C18 column (YMC-C18 column, 4.6 × 250 mm, 5-mm particle size) maintained at 35 °C. Mobile phases A and B were composed of 25 mM sodium acetate and 100% acetonitrile, respectively. A flow rate of 0.8 mL/min was used, with the following gradient program: 0–2 min, 80–75% A; 2–30 min, 75–40% A; 30–35 min, 40–80% A. The UV detector operated at a wavelength of 284 nm.

Results

Engineered E. coli **W3110 equipped with T7RNAP**

Three different designs of T7RNAP cassettes were constructed and integrated into the W3110 chromosome by traditional site-specific recombination at the *att*B sites of lambdoid coliphage HK022 (Fig. 1a). The first strain, denoted as W3110::IL5 and similar to BL21(DE3), contained a *T7 gene 1* driven by P_{LacUV5} with *lac*I/*lac*O and an additional *lac*I genes located upstream of the promoter. Second strain was W3110::L5, in which additional *lac*I at upstream of the P_{LacUV5} promoter was not presented. The third

strain, W3110::pI, was significantly different from the previous two strains because the *T7 gene1* was driven by P_{LacI}, which is widely used in iGEM (https://parts.igem.org/Part:BBa_R0010) (see detail sequence in Table S2). The newly created strains were verified by amplifying a characteristic fragment in each strain (Fig. 1b), and the results showed that all strains were successfully constructed.

The cell growth (Fig. 2a), LacZ and T7RNAP protein expressions (Fig. 2b) were compared among three engineered W3110 strains equipped with the T7RNAP. The biomass at the log-phase was slightly different between the condition with or without IPTG induction, while, at 12 h, the biomass was similar at the OD ranging from 2.2 to 2.5. Furthermore, to confirm the protein expression, SDS-PAGE was performed (Fig. 2b) and showed there were two distinct bands as comparing between the condition with or without IPTG. The identity of distinct proteins was detected by LC-MS/MS and shown in the Table 2, where a band near 100 kDa corresponded to T7RNAP, and the band between 100 to 135 kDa was identified as LacZ. Besides, the quantification was performed based on the ImageLab and shown in Table 3. Interestingly, LacZ expression was much higher in the W3110 than that in BL21(DE3) (Fig. 2b and Fig. S1). The enhancements were observed as 2.63-fold for W3110, 3.41-fold for W3110::IL5, 5.21-fold for W3110::L5, and 4.61-fold for W3110::pI (Table 3). Furthermore, the T7RNAP expression also reached up to 8.72-fold for W3110::IL5, 6.72-fold for W3110::L5 and 11.92-fold for W3110::pI, implying the promoters of P_{LacUV5} was stronger in W3110 than that in BL21(DE3) and the P_{LacI} even the strongest promoter in W3110. Except for the promoter effect, the inserted locus was supposed to be the reason that T7RNAP expression was extremely higher than that in BL21(DE3) because the locus was at HK022 *att*B site in engineered W3110 strains from 1053856–1057711 bp, while that of BL21(DE3) was around the *lac* operon site (i.e., 360473–365652 bp) (Fig. 1a). With IPTG induction, maximum T7RNAP was observed in W3110::pI while the lowest one was occurred in the W3110::L5 (Table 3, T7RNAP). The feasibility of three strains used in protein expression and chemical production was further verified in the following.

Table 2
MS/MS analysis of T7 RNA polymerase and *lacZ* gene from engineered *E. coli* W3110.

No.	Protein	Accession no.	Score	Molecular weight (Mw, Da)	Isoelectric point (pI)
1	T7-like RNA polymerase	WP_001092355	955	98.79	6.77
2	LacZ beta-galactosidase	WP_096183704	1036	116.42	5.28

Table 3
Quantification of relative protein of LacZ and T7RNAP by IPTG induction in BL21(DE3), W3110, W3110::IL5, W3110::L5, W3110::pI.

Relative protein amounts	BL21(DE3)*	W3110	W3110::IL5	W3110::L5	W3110::pI
LacZ	1.0	2.63	3.41	5.21	4.61
T7RNAP	1.0	-	8.72	6.72	11.92
*LacZ and T7RNAP in BL21(DE3) with IPTG is defined as the reference of W derivatives.					

Demonstrate lacI/lacO **regulation in the new strains by sfGFP**

Two expression vectors, the pET28a(+) plasmid with the orthogonal *lac*I/*lac*O repressor (Fig. 3a) and the pSU-T7 plasmid without repressor were used for evaluation (Fig. 3b). At first, *lac*I was included in the system to verify the *lac*I/*lac*O effect of engineered strains. As shown in Fig. 3c, there was no obvious florescence intensity in all strains without IPTG. Besides, when IPTG was induced, it displayed the similar level of sfGFP protein with the average fluorescence of 47,000 a.u. in three engineered W3110 strains, but lower expression in BL21(DE3) (i.e. 31000 a.u.). The sfGFP expression by the pSU-T7-sfGFP plasmid which lacked of *lac*I/*lac*O, the highest fluorescence intensity of 18,000 a.u. in W3110::pI without IPTG and the similar florescence intensity at 11,000 a.u. was observed in other strains (Fig. 3d). Interestingly, when IPTG was added, the specific fluorescence intensity of W3110::L5 was the highest with enhancement of 46%, 75% and 77% as compared to the BL21(DE3), W3110::IL5 and W3110::pI, respectively (Fig. 3d). As a result, *lac*I/*lac*O was the key component to regulate protein expression in W strains under IPTG induction.

Cas9 Expression And Characterization In Engineered W3110

Cas9 is a heterologous toxic protein derived from *Streptococcus pyogenes* and plays an important role in the type II CRISPR/Cas system. Two pET systems were used to express Cas9 protein: the pET21a(+) system, which includes an orthogonal *lac*I/*lac*O repressor (Fig. 4a), and the pET20b(+) system, which lacked both sequences (Fig. 4b). The protein analysis results for pET21a(+)-Cas9 showed that the protein was only produced in the presence of an inducer, and the order of protein content was BL21(DE3) ~ W3110::pI > W3110::L5 > W3110::IL5 (Fig. 4c). For pET20b(+)-Cas9, when the IPTG was absent, there were equal levels of protein expression in BL21(DE3), W3110::IL5, and W3110::L5, while that in W3110::pI was 2-fold higher than other three strains. After IPTG induction, the protein expression was increased or kept at similar level in all strains except for W3110::IL5. Among all combination of strains and conditions, W3110::pI with or without IPTG produced highest Cas9 protein, similar to those of BL21(DE3) and W3110::L5 with induction, (Fig. 4d).

To evaluate the functionality of the Cas9 protein produced from our engineered W3110 strain, an *in vitro* Cas9-RNP assay was applied. Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO, *rbcL*) was

selected as our targeted template (i.e. 1.5 k) for digestion and the sgRNA was designed to slice the DNA into two fragments (i.e. 0.6 k and 0.9 k). The results of *in vitro* cleavage showed that the *rbc*L fragment was successfully divided into two desired fragments (Fig. 4e), demonstrating that all three engineered strains produced functional Cas9 successfully.

Carbonic Anhydrase Expression And Activity In Engineered W3110 Strains

In our previous report, BL21(DE3) overexpressing carbonic anhydrase (CA) showed the arrested cell growth as compared to that without CA expression [32]. Therefore, we considered whether W3110 strain would tolerate CA overexpression via pET32a-SyCA. At first, the result of biomass in BL21(DE3) has 30% reduction at 12 h which showed the toxicity to the cell; however, newly engineered W3110 could tolerate to the CA more robustly because the reduced biomass was only 25%, 2% and 18% for W3110::IL5, W3110::L5 and W3110::pI, respectively (Fig. 5a). Due to *lac*I/*lac*O regulation in the plasmid, IPTG induction was required for CA production. The CA activity was shown in Fig. 5b. In BL21(DE3), the CA activity increased from 803 WAU to 1100 WAU as IPTG increased from 0.005 mM to 0.01 mM and kept the similar level with 0.1 mM IPTG. The CA activity of W3110:IL5 was 1400 WAU with 0.005 mM IPTG and sharply decreased to 400 WAU at that with 0.1 mM IPTG. However, the W3110::L5 and W3110:pI expressed highest CA activity with 1710 WAU at 0.01 mM IPTG and 1795 WAU at 0.005 and 0.01 mM IPTG, respectively. Therefore, the best strain for production of CA is W3110::pI because it maintains the biomass and possesses the highest CA activity with extremely low IPTG.

Conversion of lysine to cadaverine by the engineered W3110 strains

It has been reported that the W3110 strain could tolerant to 20 g/L diaminopentane (DAP) [33]. Herein, the DAP toxicity test was performed in 4 strains by the ratio of viable cell which defined as the ratio of biomass between DAP addition and without addition. It showed that the cell would dramatically reduce after the DAP of 10 g/L was added, but W3110 possess higher survival rate than BL21(DE3), in which the viable cell percent was 32.1%, 34.0%, 42.8% and 36.6% for BL21(DE3), W3110:IL5, W3110::L5 and W3110::pI (Fig. S2). Afterwards, two CadA-containing plasmids with or without *lac*I/*lac*O (i.e. pET28a(+)-CadA and pSU-T7-CadA) were transformed into four strains and the *in vivo* DAP production was conducted to verify the function of lysine decarboxylase.

The IPTG concentration was varied in the four strains harboring pET28a(+)-CadA (Fig. 6). Similar levels of CadA proteins were observed for BL21(DE3), W3110::L5 and W3110::pI, in which the CadA was expressed at IPTG concentrations from 0.1 to 0.01 mM. More CadA was expressed with 0.1 mM IPTG than that with 0.01 mM IPTG in BL21(DE3) (Fig. 6b). However, CadA in W3110::IL5 was only over-expressed with 0.1 mM IPTG, and expressed with a critical low amount of protein by 0.01 mM IPTG (Fig. 6c). Furthermore, CadA could be expressed in a critical low IPTG (i.e., 0.001 mM) in W3110::L5 (Fig. 6d), and the highest expression occurred in W3110::pI with 0.01 mM IPTG (Fig. 6e). For the *in vivo*

lysine production, lysine, IPTG, and PLP were added at 0.35 M, 0.1 mM, and 0.01 mM during the initial exponential phase (i.e., 3 h). All the strain possessed highest DAP production at 24 h as pET28a(+)-CadA was used. The highest lysine consumption for in BL21(DE3)(+) and W3110::IL5(+) was only 21.6 g/L and 18.13 g/L, respectively. However, the lysine could be more efficiently utilized in the W3110::L5 and W3110::pI with a 45 g/L and 32.41 g/L consumption of lysine. Therefore, the highest DAP production were obtained from W3110::L5(+) with 45.01 g/L lysine consumption, 32.2 g/L DAP, 1.34 g-DAP/L/h productivity and 91.73% yield at 24 h (Table 4).

Table 4
Biomass, lysine consumption, DAP titer, DAP productivity, yield of in vivo time-course with pET28a(+)-CadA plasmid and 50 g/L lysine in BL21(DE3), W3110::IL5, W3110::L5 and W3110::pI, respectively.

Strain*	Time (h)	Lysine consumption (g/L)	DAP Titer (g/L)	Productivity (g-DAP/L/h)	Yield (g/g, %)
BL21(DE3)(+)	6	9.07 ± 1.5	2.5 ± 0.1	0.42 ± 0.07	6.78 ± 1.17
	12	9.40 ± 1.7	5.2 ± 1.0	0.43 ± 0.08	13.80 ± 2.50
	24	21.60 ± 1.4	11.3 ± 1.3	0.47 ± 0.06	30.05 ± 3.95
W3110::IL5(+)	6	9.14 ± 2.4	4.0 ± 1.5	0.67 ± 0.25	11.59 ± 2.09
	12	16.67 ± 3.7	10.4 ± 1.9	0.87 ± 0.16	29.38 ± 1.80
	24	18.13 ± 0.3	13.6 ± 1.6	0.57 ± 0.07	38.12 ± 5.89
W3110::L5(+)	6	17.13 ± 1.8	11.8 ± 0.1	1.97 ± 0.02	33.62 ± 4.45
	12	40.90 ± 2.8	29.0 ± 2.0	2.42 ± 0.16	82.46 ± 7.62
	24	45.01 ± 1.2	32.2 ± 0.2	1.34 ± 0.01	91.73 ± 8.07
W3110::pI (+)	6	14.50 ± 3.7	10.12 ± 1.1	1.69 ± 0.18	29.05 ± 2.51
	12	28.45 ± 3.0	19.85 ± 2.1	1.65 ± 0.17	56.96 ± 3.05
	24	32.41 ± 2.0	22.61 ± 2.4	0.94 ± 0.10	64.89 ± 1.01

*(+) means with IPTG induction. The errors represent the standard derivation of 3 independent experiments (n = 3).

The protein expression in pSU-T7-CadA without *lacI*/*lacO* (Fig. 6f) was analyzed in four different strains. The results were similar to the Cas9 protein results, except for W3110::IL5; in addition, the leakage was lower in W3110::L5 than in BL21(DE3) and W3110::IL5 (Fig. 6g). The CadA expressions in W3110::pI(-) and (+) are similar, which indicated constitutive P_{LacI} promoter was effective. For the pSU-T7-CadA-harbored strains as shown in Table 5, *in vivo* production of DAP by lysine consumption of all strains (except for W3110::IL5) reached 80% yield at 12 h, while DAP yield decreased until 24 h, mainly due to DAP would be further utilized in the metabolic pathway. The best condition was used W3110::pI(-) to obtain 36.9 g/L DAP, 3.08 g-DAP/L/h productivity and 103.4% yield by pSU-T7-CadA. We found out that it

is reasonable that the yield value was higher than 100%. Because the lysine concentration in LB medium was approximately 1.61 g/L by HPLC analysis from the retention time (Fig S3a) and calibration curve (Fig. S3b). As comparing the results between pSU-T7-CadA and pET28a(+)-CadA, a significant enhancement in the lysine consumption rate existed by pSU-T7-CadA. The strains harboring the pSU-T7-CadA consumed up to 41.59, 37.35, 41.98 and 42.09 g/L lysine for BL21(DE3)(+), W3110::IL5(+), W3110::L5(+) and W3110::pI(+) at 24 h, while the lysine consumption of strains harboring the pET28(+)-CadA was only 21.6, 18.13, 45.01 and 32.41 g/ in the same strains. This manifested a more feasible strategy to apply the constitutive system (i.e. without the *lac*I/*lac*O regulation) in W3110 for chemical production due to higher chemical production rate and precursor consumption rate.

Table 5
Biomass, lysine consumption, DAP titer, DAP productivity, yield of in vivo time-course with pSU-T7-CadA plasmid and 50 g/L lysine in BL21(DE3), W3110::IL5, W3110::L5 and W3110::pI, respectively.

Strain	Time (h)	Lysine consumption (g/L)	DAP Titer (g/L)	Productivity (g DAP/L/h)	Yield (g/g, %)
BL21(DE3)(+)	6	24.21 ± 1.3	18.3 ± 1.2	3.05 ± 0.05	51.3 ± 1.2
	12	38.94 ± 2.2	32.4 ± 2.5	2.70 ± 0.02	90.7 ± 3.2
	24	41.59 ± 1.9	28.0 ± 1.5	1.17 ± 0.01	78.4 ± 4.3
W3110::IL5(+)	6	16.56 ± 0.6	12.8 ± 0.5	2.13 ± 0.03	35.9 ± 2.9
	12	31.14 ± 1.1	22.4 ± 1.1	2.60 ± 0.04	62.7 ± 1.6
	24	37.35 ± 1.6	23.9 ± 0.9	1.00 ± 0.01	66.9 ± 1.8
W3110::L5(+)	6	19.92 ± 1.0	15.4 ± 0.3	2.56 ± 0.02	43.1 ± 1.9
	12	37.50 ± 2.4	29.3 ± 0.5	2.44 ± 0.05	82.1 ± 1.7
	24	41.98 ± 1.4	24.9 ± 0.8	1.04 ± 0.01	69.7 ± 12.5
W3110::pI (+)	6	32.51 ± 1.1	25.4 ± 1.6	4.24 ± 0.07	71.1 ± 3.2
	12	40.22 ± 1.7	31.9 ± 2.0	2.66 ± 0.05	89.4 ± 3.5
	24	42.09 ± 1.9	26.3 ± 1.9	1.10 ± 0.02	73.7 ± 4.2
W3110::pI (-)	6	41.25 ± 1.1	36.7 ± 2.0	6.11 ± 0.11	102.8 ± 2.7
	12	43.85 ± 1.5	36.9 ± 1.3	3.08 ± 0.09	103.4 ± 3.0
	24	44.69 ± 0.9	31.4 ± 2.8	1.31 ± 0.02	88.0 ± 2.2

*(+) means with IPTG induction; (-) means without IPTG induction. The errors represent the standard derivation of 3 independent experiments (n = 3).

Discussion

Tunable protein expression is crucial for synthetic and system biology. One of the powerful tools is the T7RNAP and its orthogonal T7 promoter. T7RNAP originating from the bacteriophage T7 elongates the RNA at a rate approximately 5-fold faster than that of *E. coli* native RNA polymerase with specific recognition for the T7 promoter [34]. Therefore, the T7RNAP-mediated protein expression system was first applied in BL21(DE3) and showed that the chromosome-based *T7 gene 1* is more suitable for toxic gene expression from plasmids by preventing instability from the strong orthogonality of T7 system when compared to the plasmid-driven T7 *gene 1* [35]. W3110 is one of the eminent *E. coli* strain and has been reported with high capability of withstanding different toxic chemicals [21–22]. Consequently, W3110 is a suitable strain to be equipped with the T7RNAP onto the chromosome. For examples, Liu and his colleagues has constructed the W3110(DE3), which entirely encompasses the same genetic design of BL21(DE3), to produce D-xylonic acid [36]. In this study, we provide three new W3110 strains equipped with different cassette of the T7RNAP as W3110::IL5, W3110::L5 and W3110::pI, which is achieved by the HK022 site-specific recombination, and displays its feasibility to produce heterologous protein and chemical production. Besides, the IPTG and *lac*O/*lac*I effect was further been elucidated.

From our results, W3110 showed a strong *lac* operon because the *lac*Z was overexpressed than that in BL21(DE3) when IPTG was expressed (Table 3), which was the first observation in our best knowledge. After addition of the T7RNAP cassette, the LacZ was further increased in expression, which was supposed that the partition of *lac*I from the *lac*Z to T7RNAP enhanced the *lac*Z expression. LacI partition has already been proven to occur and applied in different applications. Cranenburgh and his coworkers developed a plasmid stabilization system, in which the plasmid containing multiple *lac*O sequence must be maintained in the cell to compete the *lac*I binding to prevent the binding to an essential gene [37]. On the other hand, in our design, the T7RNAP was immensely overexpressed in W3110 than that in BL21(DE3), which was elucidated by the insertion locus. Actually, the expression of insertion gene will be influenced by the surrounding DNA context which may contain a promoter, RBS or even a terminator to influence the down-stream gene expression [38]. In such context, it has been supposed that the wield behavior of W3110::IL5 with higher T7RNAP than that in W3110::L5 was also contributed by the surrounding DNA context to influence the *lac*I expression, which further affect the T7RNAP expression. Moreover, recently, the chromosome-base gene expression has been detailed elucidated in the aspect of the gene locus and it shown that the gene expression level must be determined by the easiness to relocate the gene to the nucleoid periphery [39], which further supported our explanation.

For the different proteins expression, by using a *lac*I/*lac*O containing plasmid, it shows that all the expression could only occur as IPTG exists, while, as the plasmid without *lac*I/*lac*O was utilized, the expression could both be observed in absence or presence of IPTG. Additional *lac*I in plasmid (i.e. pET plasmid, except for pET20b) could repress the T7RNAP expression tightly to reduce the leakage expression [6]. As the *lac*I/*lac*O was not encompassed in the plasmid (i.e. pSU or pET20b), the leakage expression is observed, but the protein expression with induction was still higher than that without induction in BL21(DE3), W3110::IL5 and W3110::L5, which is reasonable because the *lac*I on the chromosome could still sufficiently repress the P_{LacUV5} with native *lac* RBS to express T7RNAP.

Intriguingly, in pSU plasmid series, W3110::pI could express similar amount of heterologous protein with or without IPTG, which was attributed to the highly leakage level of T7RNAP. Actually, to establish a tightly regulated chromosome-based gene expression is intricate due to the difficulty to well-balance the promoter strength, *lac*I and *lac*O amount [40]. Besides, with the similar promoter strength of P_{LacI} and P_{LacUV5} [6], the strength of RBS dominates the leakage expression of T7RNAP, where the B0034 RBS was extremely stronger than the *lac* native RBS (Fig. S4).The *in vivo* DAP production was also affected and displayed higher DAP yield in a short-term transformation (i.e. 6 and 12 h) as the plasmid without *lac*I/*lac*O was used, while the time used for highest production by plasmid with *lac*I/*lac*O must be extended to 24 h.

IPTG dose response is another point that have to be considered in an inducible system as pET with *lac*I/*lac*O was used [41]. In our results, W3110::IL5 must at least be induced by 0.1 mM to obtain the enough recombinant protein, while W3110::L5 and W3110:pI only used 0.01 mM to induce sufficient protein amount. Even with the similarly high expression of recombinant protein, the solubility would further affect the function of the recombinant protein [42], supporting the CA result where the optimal activity was occurred with 0.01 mM IPTG in the three engineered W3110 strains. Furthermore, the high orthogonality of T7 system was reported as an energy-intensive process [35], leading to less energy in folding the recombinant protein [43]. The *in vivo* production of DAP by W3110::pI harboring pSU-T7-CadA produced higher DAP without induction, primarily due to the cellular energy was more concentrated for lysine and DAP transportation as well as PLP regeneration [44–45].

Conclusion

In this study, the characterization of three engineered W3110-equipped with T7RNAP was accomplished as well as the difference of the *lac*I/*lac*O regulation between W3110 and BL21(DE3) strains was proposed. Among all, the most robust one, W3110::pI strain with a T7RNAP driven by the promoter P_{LacI}, enables effective, stable and constitutive production of several recombinant proteins and chemicals while strain W3110::L5 showed the similar capacity to produce the recombinant protein, but higher capacity to produce chemical as compared to the commercial BL21(DE3). Even though the W3110::IL5 does not behave efficiently as the other strains, it provides the new insights into the difference of Lac operon between B and W derivatives. Therefore, we not only provided a cost-effective, robust and novel engineered W3110 strain as a cell factory for recombinant technology, but also presented more understandings of different *E. coli* strains.

Declarations

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Author contributions

ISN and WWT conceived and designed research. WWT and SIT conducted experiments. ISN, WWT and SIT analyzed data and wrote the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate

All of the authors have read and agreed to the ethics in publishing this manuscript.

Consent for publication

The authors provided consent for publishing this manuscript.

Competing interests: The authors declare that they have no competing interests.

Availability of data and material: We agree to the terms of the BioMed Central Copyright and License Agreement.

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Figures

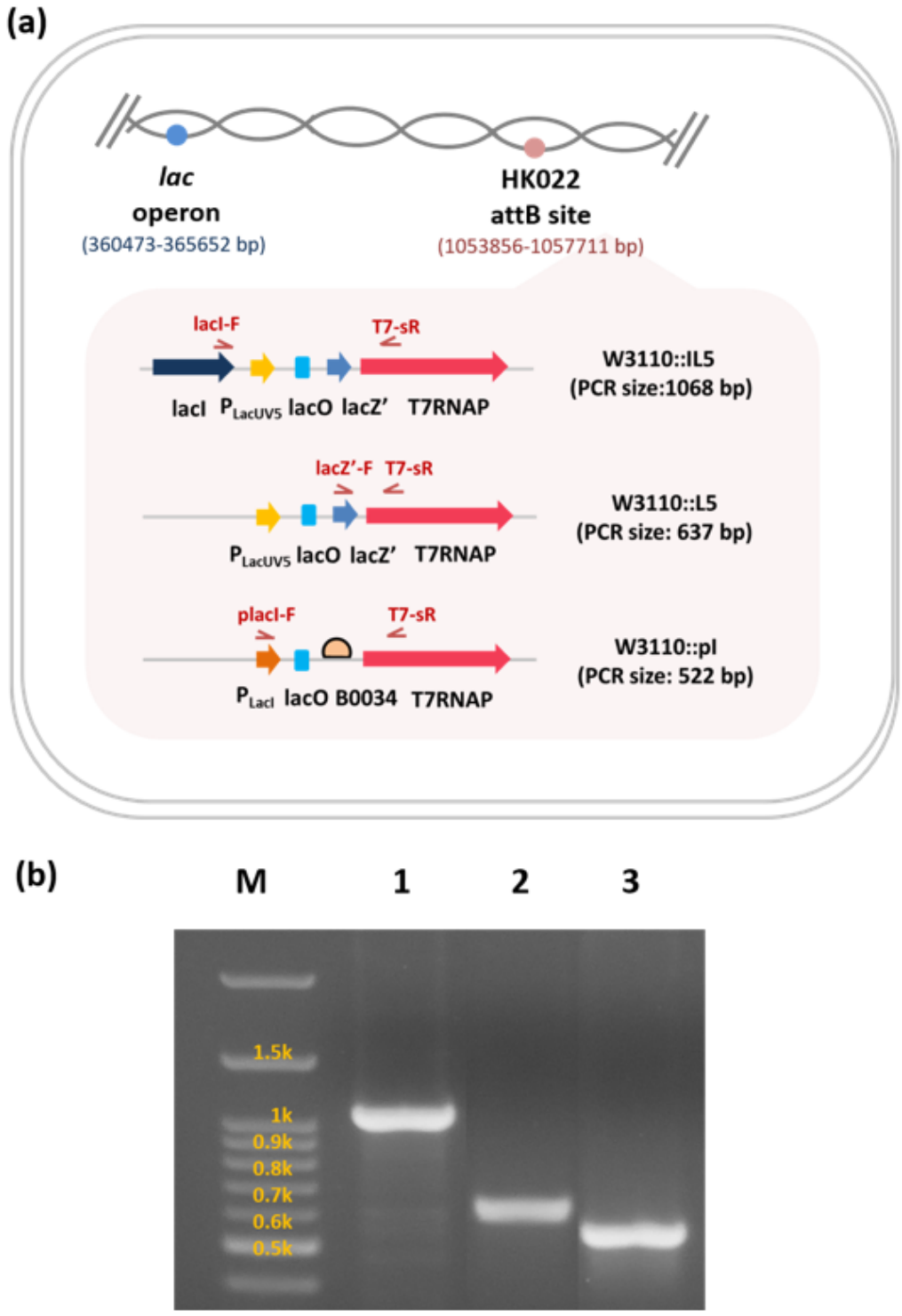

Figure 1

Scheme diagram of the engineered W3110 strain with chromosome-equipped T7RNAP (a) genetic design of engineered T7RNAP in E. coli W3110. Integration was achieved by site-specific recombination at the HK022 attB site. Three engineered T7RNAP expression strains were created. The strain W3110::IL5 has a lacI-PLacUV5-lacZ'-T7RNAP copy from BL21(DE3), and W3110::L5 lacks an additional integrated lacI. W3110::pI, in which T7RNAP is driven by the PLacI without additional repressor and lacZ' sequence near

the promoter. The characteristic fragments of each strain were labeled by primers and result of confirmation was showed in (b): lane 1: PCR with primer lacI-F and T7-sR of W3110::IL5, lane 2: PCR with primer lacZ'-F and T7-sR of W3110::L5, lane 3: PCR with primer PlacI-F and T7-sR of W3110::pI. The desired fragment of lacI-F and T7-sR is 1068 bp, lacZ'-F and T7-sR is 637 bp and 522 bp for PlacI-F and T7-sR. M: 100 bp marker

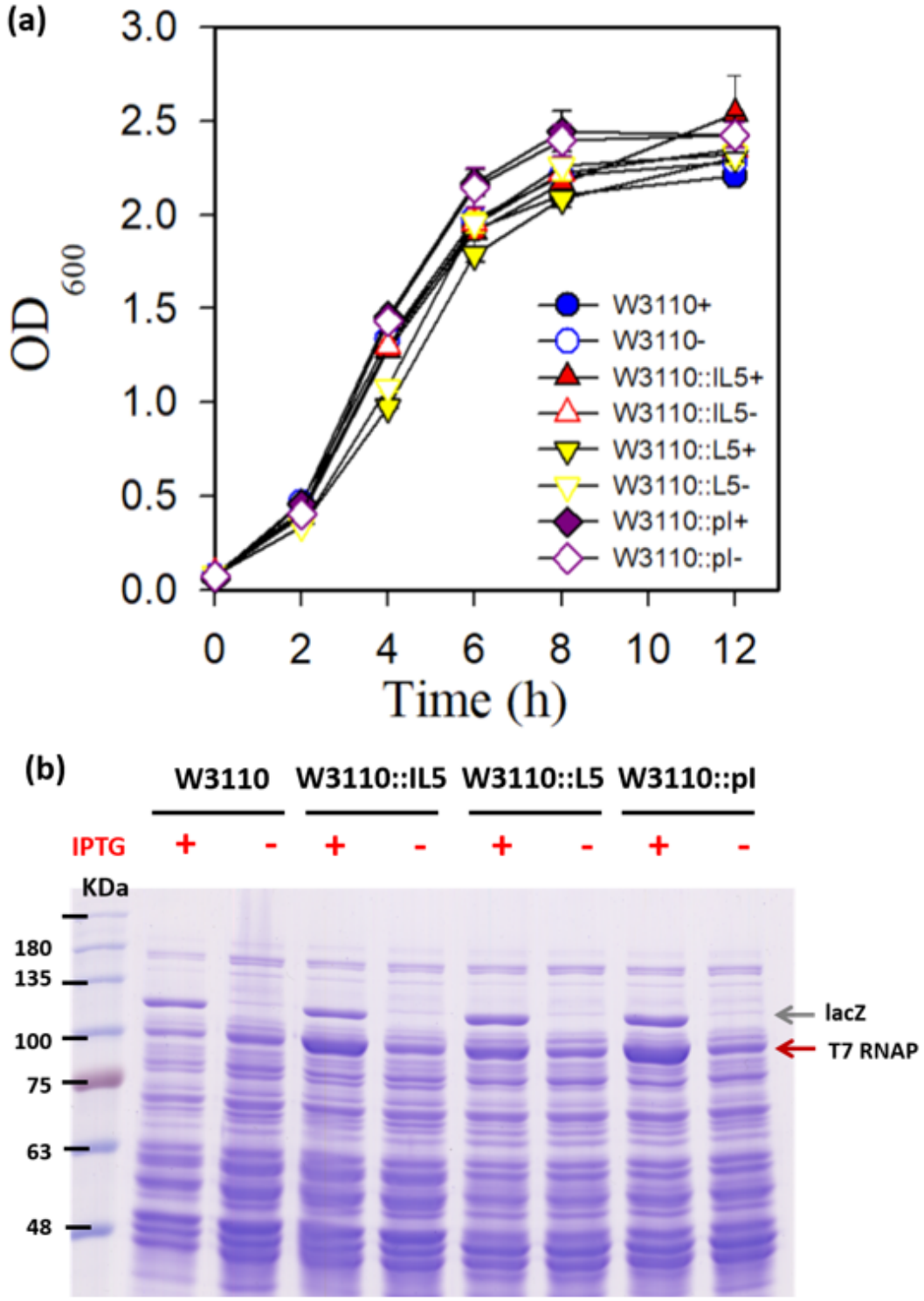

Figure 2

Cell growth and protein expression analysis of four E. coli strains with and without induction by 0.1 mM IPTG. (a) Cell growth and (b) SDS-PAGE for W3110, W3110::IL5, W3110::L5, and W3110::pI. The LacZ protein is approximately 116.4 kDa, as indicated by the gray arrow. The T7RNAP was approximately 98.8 kDa, as indicated by the red arrow.

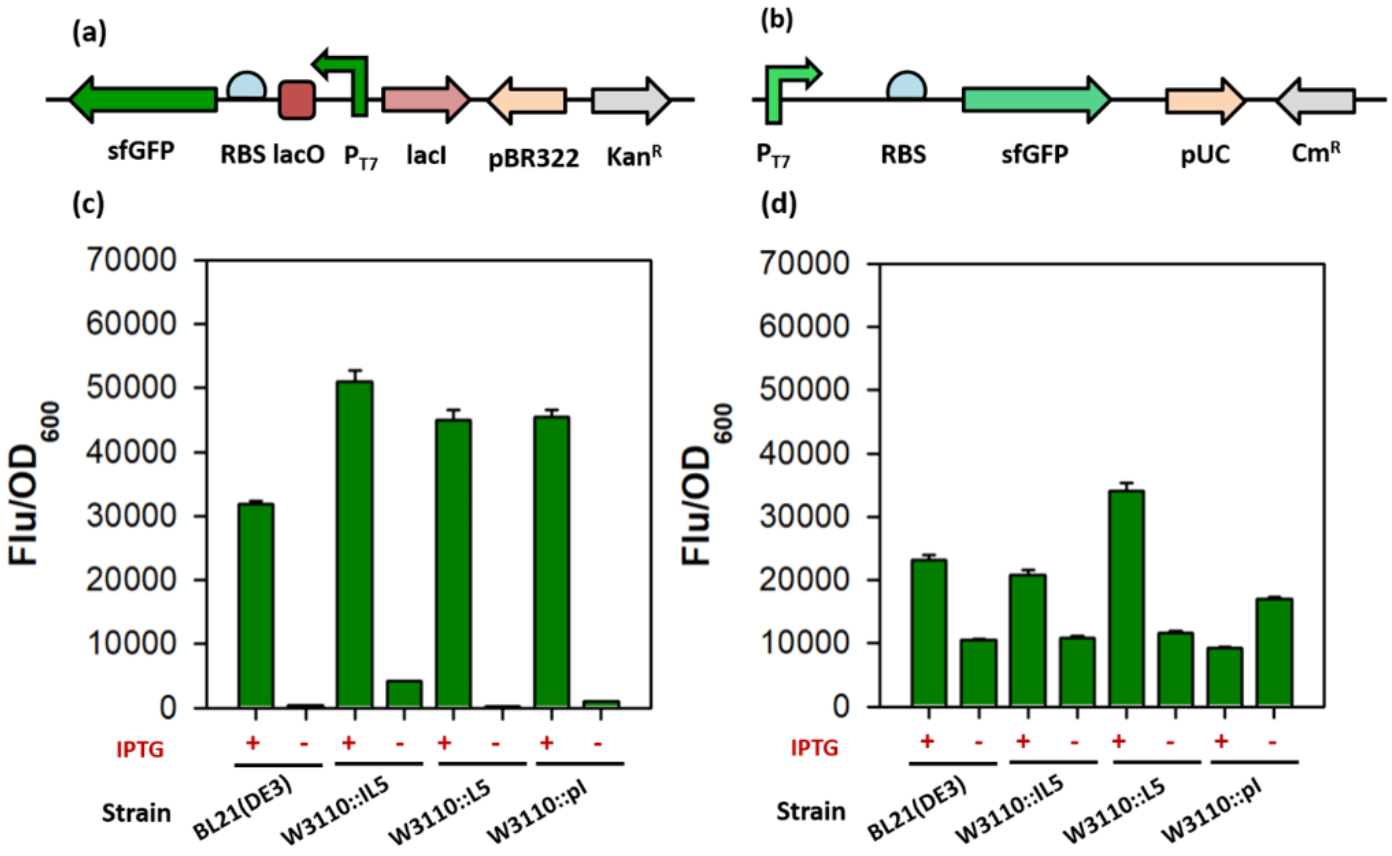

Figure 3

Comparison of sfGFP synthesis in the designed T7RNAP-carrying strains, with BL21(DE3) as a reference. sfGFP expressed from pET28a(+)-sfGFP plasmid (a) and pSU-T7-sfGFP plasmid (b). The fluorescence was analyzed by a microreader at 12 h, with excitation and emission wavelengths of 480 and 510 nm, respectively. (+) denotes IPTG induction and (-) denotes no IPTG induction.

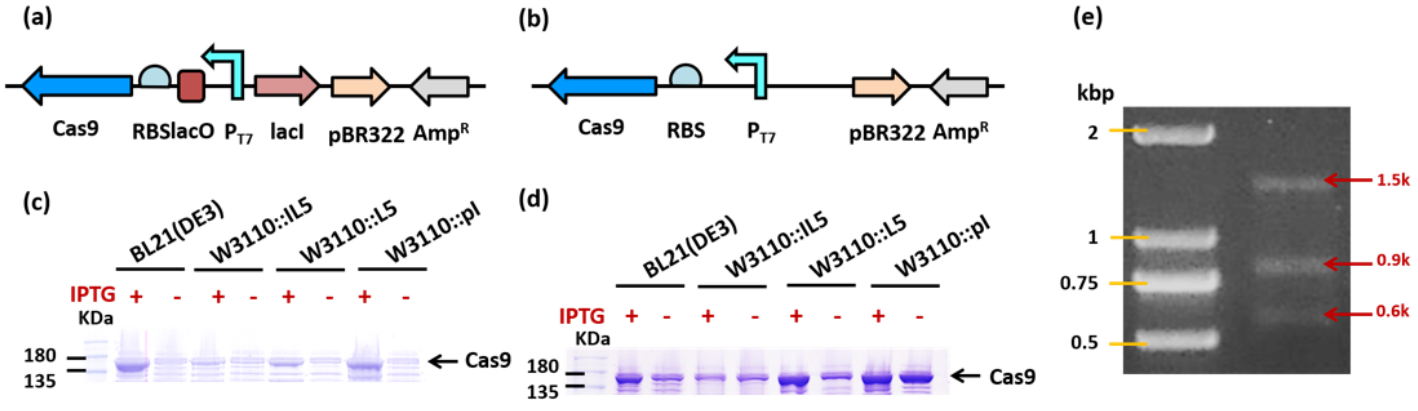

Figure 4

Functional Cas9 protein expression in engineered W3110 strains with chromosome based T7RNAP and BL21(DE3) as a control. Cas9 protein expressed from inducible pET21a(+)-Cas9 plasmid (a) and constitutive pET20b-Cas9 plasmid (b). Results are shown in (c) for pET21a-Cas9, and (d) for pET20b(+)-Cas9. The cells were harvested at 12 h after induction, and the whole cell protein expression of Cas9 was analyzed by 8% (w/v) SDS-PAGE. Cas9 has a size of 151.6 kDa, as indicated by the black arrow. (+) denotes IPTG induction and (-) denotes no IPTG induction. DNA gel results of an in vitro digestion assay with Cas9-RNP. The original size of the rbcL gene was 1.5 kbp, and the target site was designed to produce fragments of 0.6 and 0.9 kbp after Cas9-RNP digestion (e).

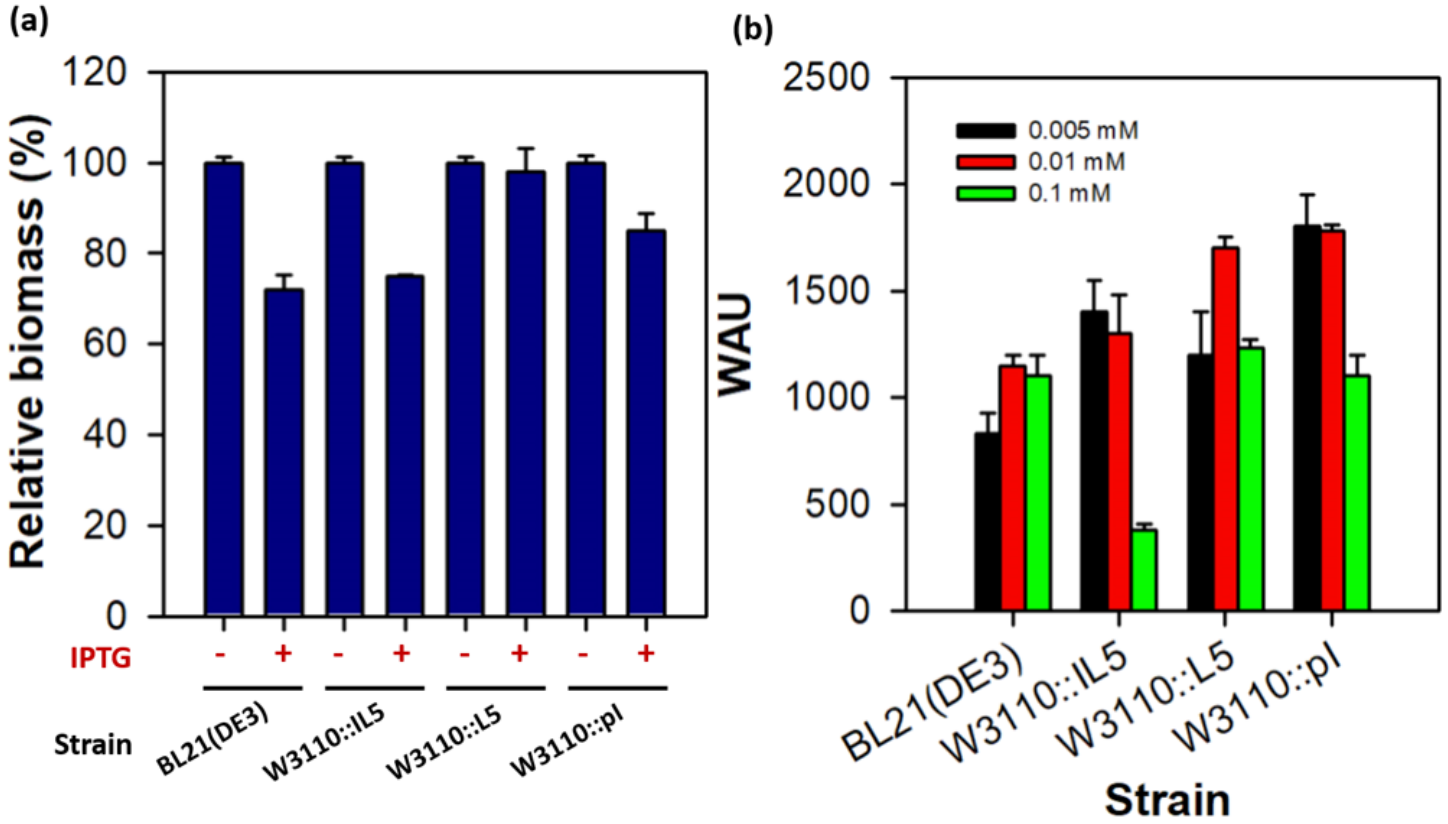

Figure 5

The cell biomass and enzyme activity of pET32a-SyCA in different engineered E. coli strains. (a) The relative biomass of different strains harboring pET32a-SyCA with or without 0.1 mM IPTG induction. (+) meant with IPTG and (-) meant without IPTG. Each strain without induction was chosen as standard respectively. (b) The activity of crude enzyme of pET32a-SyCA with 0.005, 0.01, 0.1 mM IPTG in different strains determined by Wilbur-Anderson assay.

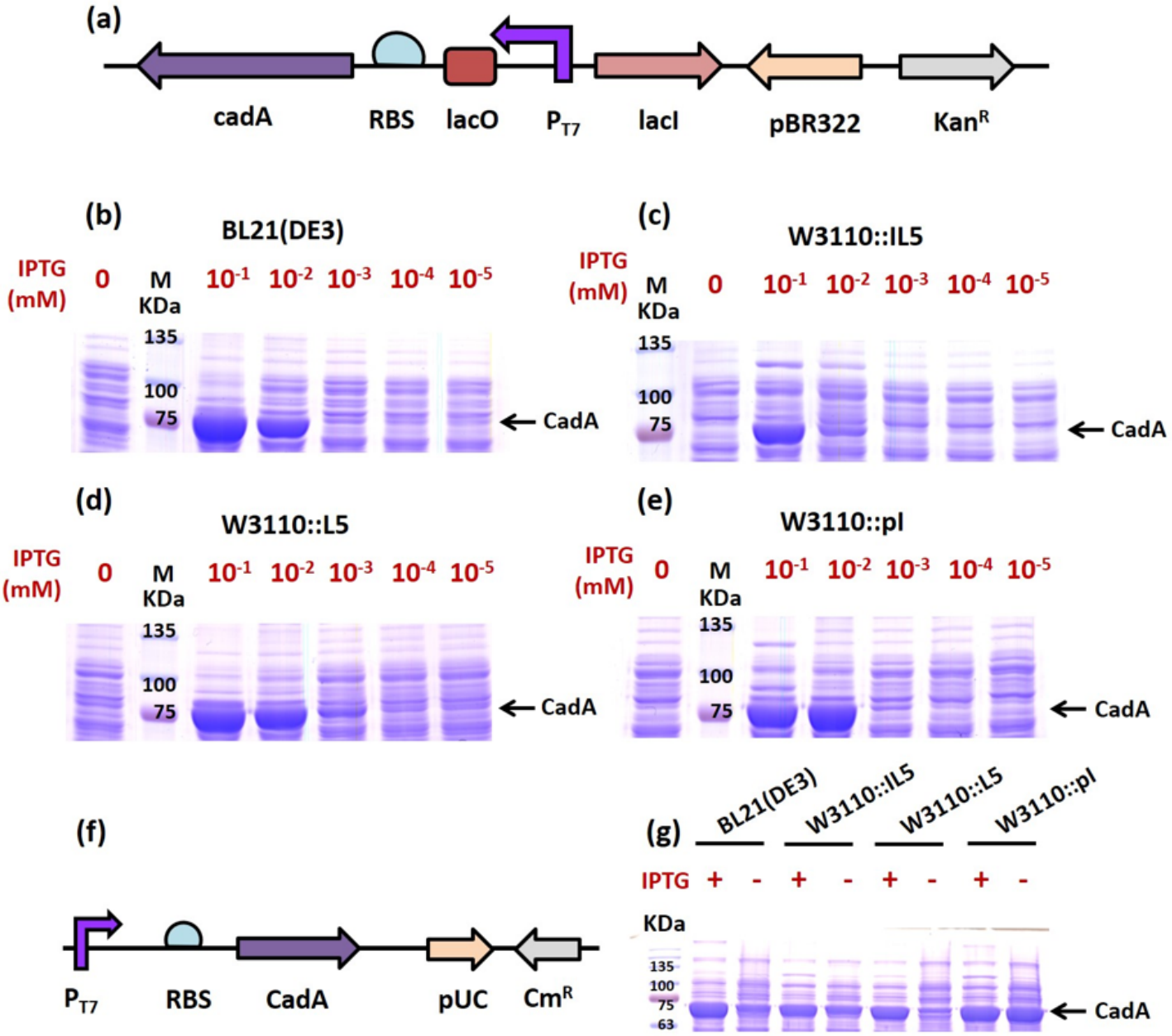

Figure 6

SDS-PAGE analysis of CadA protein expression from pET28a(+)-CadA plasmid (a) with 0, 10-1, 10-2, 10-3, 10-4 to 10-5 mM IPTG for the designed T7RNAP carrier strains. Results are shown for BL21(DE3) (b), W3110::L5 (c), W3110::IL5 (d), and W3110::pI (e). CadA protein expressed from pSU-T7-CadA plasmid (f). Whole cell protein expression of CadA from pSU-T7-CadA in different strains with or without 0.1 mM IPTG was analyzed by 8% (w/v) SDS-PAGE (g). CadA has a size of 78.76 kDa, as indicated by the purple arrow. (+) denotes IPTG induction and (-) denotes no IPTG induction.

Supplementary Files

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- 2020W3110supporting.docx