Construction of a plasmid addiction system using *grpE* as selection marker in *Escherichia coli* and its application in phloroglucinol biosynthesis

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Abstract

Microbial synthesis of commodity chemicals often be conducted in recombinant plasmid-based expression systems, in which plasmids play pivotal roles on productivity. The recombinant plasmids always encounter instability, leading to losses in product recovery of entire process. To maintain the stability of plasmids, several mechanisms have been evolved. Plasmid addition system, selectively killing plasmid-free cells, is regard as a useful strategy to improve the proportion of plasmid-containing cells. In this study, a novel plasmid addition system using an essential gene grpE that encodes a molecular cochaperone as selection marker, avoiding use of antibiotics, was constructed in Escherichia coli . The solo copy of grpE gene on the chromosome was knocked out and relocated on multicopy plasmids. The generated strains can maintain high ratio of plasmid-harboring cells without antibiotics supplementation in mineral salts media and exhibit improved cell growth and increased tolerance to phloroglucinol. Using this system in phloroglucinol synthesis, it could significantly increase the phloroglucinol titer from 0.75 g/L to 1.26 g/L, which was further increased to 1.78 g/L when biotin-[acetyl-CoA-carboxylase] ligase BirA was overexpressed. It can be expected that this system will be a powerful tool for microbial manufacture of important chemicals in E . coli .

Key Points

- A novel *grpE* based plasmid addition system was constructed in *Escherichia coli*.
- Biosynthesis of phloroglucinol was remarkably boosted using this system.
- Phloroglucinol titer was further improved by overexpression of *birA*.

Introduction

The constantly growing demand for renewable green chemicals and fuels has stimulated great development of synthetic biology. Metabolic engineering of microorganisms by rewiring cells' metabolic flux towards desirable chemicals has been proved to be a promising strategy (Palazzotto et al. 2019; Wang et al. 2018; Westbrook et al. 2019). Microbial syntheses of important biobased chemicals often be conducted in well-established hosts such as *Escherichia coli* with plasmid-based expression systems , which highly rely on foreign genetic information encrypted in the plasmids (Kroll et al. 2010). Plasmids still supply some of the most versatile systems for the expression of foreign genes, which seriously depend on the plasmid stability and the copy number. Antibiotic resistance selection markers are always introduced to most of frequently used cloning or expression plasmids and antibiotics must be added to the media for plasmid maintenance as well as for easy transfer to the hosts (Lau et al. 2013; Summers 1991). Nevertheless, plasmid instabilities could not be totally avoided (Wang et al. 2020). Plasmid-free cells could decrease the product titer and the profitability of the whole process (De Gelder et al. 2007; San Millan and MacLean 2017). Moreover, utilization of antibiotics is feasible at the laboratory scale, but are not applicable in the industrial procedures owing to the high costs and environmental issues. Chromosomal gene integration is an alternative used for decades to stabilize foreign genetic information.

However, it often results to low recombinant protein levels or poor product yields compared to the use of (multi-copy) plasmids (Li et al. 2019).

Several mechanisms for plasmids stability have been evolved, one of which, plasmid addiction system (PAS), prevents the survival of plasmid-free cells (Kroll et al. 2011; Tsang 2017; Zielenkiewicz and Ceglowski 2001). PASs can be grouped into three major categories: (i) toxin/antitoxin-based systems, (ii) metabolism-based systems and (iii) operator repressor titration systems (Cranenburgh et al. 2004; Kawano et al. 2007; Kroll et al. 2010; Kroll et al. 2009; Ogura and Hiraga 1983). Various PASs have been successfully constructed. However encountering many problems such as invalidation during prolonged culture growth (Cooper and Heinemann 2000) , survival of plasmid-free cells by assimilating nutrition released from plasmid-containing cells (Gao et al. 2014) and so on. Another strategy of PAS is to knock-out essential genes from the chromosomes and restore these genetic deficiencies on plasmids. Recently, two PASs using essential genes *ispH* and *lpxA* as selection marker respectively were constructed and were applied in the biosynthesis of different chemicals (Kroll et al. 2011; Zhao et al. 2019). Nevertheless, these genes are based on catabolism genes whose manipulation would cause metabolic imbalance of the host cells. To overcome these problems, an essential gene *grpE* was chosen for PAS construction. *grpE* encodes a cochaperone, functioning as the nucleotide exchange factor for the molecular chaperone DnaK and supporting bacterial growth at all temperatures (Ang and Georgopoulos 1989; Harrison et al. 1997). Generally, molecular chaperones protect cellular homeostasis of the host cells by promoting protein folding and disaggregation. It could be hypothesized that *grpE* expression would be helpful in response to environmental stresses and to biosynthesis of toxic products.

Phloroglucinol (PG), a natural phenolic substance, can serve as antioxidant and low toxic smooth muscle antispasmodic (Cahen 1962; Kang et al. 2006; Singh et al. 2009). PG represents the scaffold of more than 700 natural products and has been demonstrated as building block for synthesis of many valuable chemicals traditionally derived from fossil resources including some important energy materials or explosives, such as 2,4,6-trinitro-1,3,5-benzenetriol and 1,3,5-triamino-2,4,6-trinitrobenzene (Hoffman and Fontes 2010; Singh et al. 2010; Wang et al. 2007; Yang and Cao 2012). Traditionally, PG was manufactured by chemical processes which always encountered problems related to safety, environmental pollution or low yield (Singh et al. 2009). Recently, natural PG was detected in the culture extract of *Pseudomonas fluorescens* Pf-5 (Nowak-Thompson et al. 1994) and direct biosynthesis of PG from glucose in *E. coli* has been achieved by solo expression of *phlD*, a gene located in the *phlACBDE* cluster corresponding for the synthesis of 2,4-diacetylphloroglucinol in *P. fluorescens* Pf-5 (Achkar et al. 2005; Nowak-Thompson et al. 1994; Siddiqui and Shahid Shaukat 2003). PhlD is a novel type III polyketide synthase, catalyzing the cyclization of three malonyl-CoA precursors to yield one PG as well as three CO_2 (Zha et al. 2006). Although the titers of PG in *E. coli* were remarkably enhanced by different genetic and metabolic engineering approaches (Cao et al. 2011; Liu et al. 2016; Rao et al. 2013; Zha et al. 2008; Zhang et al. 2017), the inherent toxicity of PG can enormously inhibit growth and viability of the hosts and limit the titers during bacterial fermentation. Hitherto microbially synthesizing PG are still far away from industrial profitability.

The main objective of this study was to construct a novel PAS using an essential cochaperone encoding gene *grpE* as selection marker in *E. coli* BL21(DE3) and applied in synthesis of biobased chemicals in cheap mineral salts media without antibiotics addition. To verify the efficiency of the novel PAS in excluding competing plasmid-free cells and PG synthesis, PG producing pathway was reconstituted in the novel *grpE* based PAS and the bacterial growth and PG production was investigated in a cheap mineral salts medium without antibiotics stress.

Materials And Methods

Strains, plasmids, primers, and media

E. coli CC118 served as the host strain for amplification of pRE112 suicide plasmid and those pRE112 derived. *E. coli* DH5α served as the host strain for gene cloning except pRE112 related plasmids. *E. coli* χ7213 was used for the preparation of the donor of conjugation transfer. *E. coli* BL21(DE3) was used for the preparation of the recipient of conjugation transfer. *E. coli* strains were grown routinely in lysogeny broth (LB) or LB agar plates. Screening for the suicide plasmid lost recombinants, NaCl-free LB agar plates containing 10% (w/v) sucrose were used. For PG production, the recombinant strains were cultured in an mineral salts medium described in (Zhang et al. 2017) with minor changes which contained 20.0 g/L glucose, 12.8 g/L $K_2HPO_4{\cdot}3H_2O$, 1.2 g/L citric acid monohydrate, 3.0 g/L $(NH_4)_2SO_4$, 0.3 g/L ferric ammonium citrate, 2 mL of 1M MgSO4, 1 mL of trace element solution containing $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ 0.35 g/L, $ZnSO_4{\cdot}7H_2O$ 2.87 g/L, H_3BO_4 2.47 g/L, $CuSO_4{\cdot}5H_2O$ 0.25 g/L, $CaCl_2{\cdot}2H_2O$ 2.94 g/L, $MnCl_2{\cdot}4H_2O$ 1.58 g/L, $CoCl_2{\cdot}6H_2O$ 0.48 g/L, $NiCl_2{\cdot}6H_2O$ 0.48 g/L and Na_2SeO_3 0.35 g/L and 1 mL of vitamin solution containing thiamine pyrophosphate 1.0 mg/mL and biotin 1.0 mg/mL. Except where specifically mentioned, appropriate selective compounds were added to the media with final concentrations at 100 μg/mL of ampicillin (Amp), or 34 μg/mL of chloramphenicol (Cm), or 50 μg/mL of kanamycin (Kan), or 50 μg/mL of DL-α,ε-diaminopimelic acid (DAP).

The *grpE* knockout cassette (*grpE*$_k$) consisted of two DNA segments, upstream (5'-arm) and downstream (3'-arm) of *grpE* coding sequence respectively, which were amplified by common PCR using grpE$_k$-F/grpE$_k$-RV0 and grpE$_k$-F0/greE$_k$-RV as primers and the genomic DNA of *E. coli* BL21(DE3) as template and was generated by overlap PCR using grpE$_k$-F/grpE$_k$-RV as primers and those two PCR products as template. The *grpE* expression cassette (*grpE*$_e$) and the *birA* gene were PCR amplified from the genomic DNA of *E. coli* BL21(DE3) using grpE$_e$-F/grpE$_e$-RV and birA-F/birA-RV as primers, respectively. The *phlD-marA* fragment (*pmf*) was PCR amplified from pET-phlDmarA using pmf-F/pmf-RV as primers. These PCR products were purified and ligated to pEasy-Blunt cloning vector to form pEasyB-grpE$_k$, pEasyB-grpE$_e$, pEasyB-birA and pEasyB-pmf respectively, which were confirmed by DNA sequencing. *grpE*$_k$ was inserted into pRE112 to form pRE112-grpE$_k$ via *Sac*I/*Kpn*I. A cloning plasmid pUC57S-grpE$_m$ carrying mutated *grpE* expression cassette (*grpE*$_m$) in which the codons of *grpE* were synonymously mutated was purchased from Nanjing GenScript Biotech Co., Ltd. (Nanjing, China). *grpE*$_m$ was inserted into pKD46 via

*Nco*I to generate pKD46-grpE$_m$. *pmf* was inserted into pA-accADBC to generate pPGN-1 via *Not*I/*Afl*II. *grpE$_e$* was inserted into pPGN-1 via *Xho*I/*Pac*I to construct pPGNE-1. Biotin-[acetyl-CoA-carboxylase] ligase coding gene *birA* was inserted into pPGNE-1 by *Stu*I/*Not*I to generate pPGNE-2.

All the strains and the plasmids used in this work were listed in Table 1. The oligonucleotides used in this study and the plasmid maps were attached in the Supplemental Table S1 and Supplemental Fig. S1, respectively.

DNA manipulations and transformation

DNA manipulations were carried out using routine methods. *E. coli* plasmids were purified using Monarch™ Plasmid Miniprep Kit (New England Biolabs, USA). PCR products were recovered using Monarch™ DNA Gel Extraction Kit (New England Biolabs, USA) or Monarch™ PCR & DNA Cleanup Kit (New England Biolabs, USA). Restriction endonuclease digestions and DNA ligations were performed according to the manufacturer's instructions. PCR amplifications were performed in 20 or 50 μL of reaction systems containing 1× PrimeSTAR® max premix (Takara, China), primers 0.5 μM each, and 1.0 μL of DNA template (about 100 ng). *E. coli* strains were transformed with plasmid DNA using the $CaCl_2$ method according to Sambrook *et al* (Sambrook and Russell 2001), or with minor changes which would be described as necessary.

Construction of *ΔgrpE* mutant of *E. coli* BL21(DE3)

Briefly, the *grpE* knockout strain of *E. coli* BL21(DE3) was constructed by allelic exchange method (Edwards et al. 1998). *E. coli* BL21(DE3) and *E. coli* χ7213 were respectively transformed by pKD46-grpE$_m$ and pRE112-grpE$_k$ to generate the recipient R-0 and the donor D-0 of conjugation. R-0 and D-0 were separately grown in LB broth containing appropriate antibiotics or DAP with shaking at 30°C until an $OD_{600} \approx 0.6$-0.7 was reached. 1.5 mL of the two cultures were collected and mixed by brief vortex. The cells of the mixture were harvested by centrifugation, washed twice with 500 μL of double-distilled water, resuspended in 50 μL of 1% (w/v) DAP solution, spread on a LB plate without any antibiotics, and incubated at 30°C for 18 h. The transconjugants were harvested, washed twice with double-distilled water, spread on LB plates with Cm and Amp and incubated at 30°C overnight to screen for clones that acquired Cm resistance via a single crossover event. Twenty of the growing clones were confirmed by PCR using gck-F/gck-RV as primers, and then replica-plated onto LB agar plates supplemented with Cm and Amp and NaCl-free LB-10% sucrose agar plates supplemented with Amp. Those colonies which were sensitive to Cm and tolerant of sucrose and Amp were further purified on NaCl-free LB-10% sucrose agar plates. Twenty of these colonies were randomly selected and their genotypes were examined by PCR using gck-F/gck-RV as primers. The resulting fragments were sequenced. One of the genotype-confirmed strains was assigned as PGH-0 and was selected for further study.

Effects of *grpE* relocation on plasmid

To investigate the effects of *grpE* relocation on plasmid, the growth profiles of PGH-0 and BL21(DE3) with or without exogenous PG stress were examined. The selected strains were precultured in 5.0 mL of LB broth without any antibiotics. The precultures were inoculated into 250 mL baffled Erlenmeyer flasks containing 50 mL of mineral salts media supplemented with or without PG at an initial OD_{600} = 0.1. Due to the limited solubility of PG in water (1.22 g PG/100 mL water), mother solution of PG was prepared by dissolving PG in dimethyl sulfoxide (DMSO) at a concentration of 250 g/L. 500 μL of PG mother solution was added the medium to make a final concentration of 2.5 g/L. Fermentation broths supplemented with 500 μL of water or DMSO were used as controls and vehicles, respectively. The cultures were incubated at 30℃ for 36 h and sampled every 4 h, and the cell densities of the cultures were recorded.

Construction of recombinant PG synthesizing strains

The recombinant PG synthesizing strains were constructed by transforming the host strains via the routine $CaCl_2$ method with minor changes (Sambrook and Russell 2001): (i) the host strains were grown in LB broth at 30℃ before being rendered to competent cells; (ii) the transformants were selected on LB agar plates containing Cm at 42℃ overnight. pKD46 (containing a temperature sensitive R101 replication origin) derived plasmids could not replicate under higher temperature. To confirm the loss of pKD46-$grpE_m$ in *E. coli* PGNE-1 and *E. coli* PGNE-2 after being incubated at 42℃ overnight, PGNE-1 and PGNE-2 were respectively inoculated into 5.0 mL LB broth containing Amp or Cm and cultured with shaking for 14 h. The growth of the strains under different antibiotic stresses were investigated.

PG synthesis in shaking flask cultivation

PG synthesis was carried out in 250 mL baffled Erlenmeyer flasks containing 50 mL of mineral salts fermentation media supplemented with or without Cm using PGN-1, PGNE-1, and PGNE-2 as producers. Single colony of each strain was inoculated in 5.0 mL LB broth overnight. The cultures were used as the seeds. The seed cultures were inoculated to the fermentation medium at an initial OD_{600} = 0.1. The cultures were incubated in a gyratory shaker at 37°C and 200 rpm until an $OD_{600} \approx 0.7$ was reached. A final concentration of 0.2 mM IPTG was added to the media, and the cells were induced at 30°C for 24 h. Cell density and PG production were monitored during the whole fermentation.

Analytical methods

Cell densities were determined by measuring the absorbance at 600 nm (OD_{600}) with Cary 50 UV–Vis spectrophotometer (Varian, USA). Samples were diluted with the appropriate medium as needed to ensure an absorbance at 0.20-0.80. The concentrations of PG in the media supernatant were quantified by cinnamaldehyde colorimetric assay. Briefly, cinnamaldehyde was dissolved in ethanol/HCl (v:v = 3:1) to

make final concentration of 10 mg/L. 5 μL of culture was added to 1 mL of cinnamaldehyde solution, fully mixed and incubated at room temperature for 15 minutes. Absorbance of the solution was measured at 446 nm (OD_{446}) using Cary 50 UV–Vis spectrophotometer. The concentration of phloroglucinol was determined using a standard curve. All the assays in this study were performed in triplet except for those specifically mentioned.

Results

Construction of expression host with chromatinic *grpE* disrupted

A chromatinic *ΔgrpE* deletion mutant of BL21(DE3) named PGH-0, harboring supplementary copies of allelic $grpE_m$ on the helper plasmid pKD46-$grpE_m$, was constructed by allelic exchange. Considering the high efficiency of gene knockout in *E. coli* BL21(DE3), allelic exchange by conjugative transfer based on pRE112 suicide plasmid was used to disrupt the *grpE* gene. pRE112 replication initiation absolutely needs a phage protein Pir absent in most of *E. coli* strains including BL21(DE3) but present in χ7213 and C118. The principle of the construction is shown in Fig. 1b. A hybrid DNA fragment containing 5'-arm and 3'-arm adjacent to the coding sequence of *grpE* was obtained by PCR and inserted into pRE112 to generate pRE112-$grpE_k$ which would integrate into the genome of recipient strain by single crossover and endue the recipient strain with Cm resistance. Since deletion of the solo copy of *grpE* on the chromosome is lethal, extra copies of *grpE* allele must be transferred to the host cells before *grpE* knockout. The *grpE* allele $grpE_m$ was placed on the temperature sensitive helper plasmid pKD46-$grpE_m$ which conferred Amp resistance to the host cells, supported the vitality of the host cells at lower temperature (30℃ for example), and could be cured at 42℃. To avoid the allelic exchanges between linear pRE112-$grpE_k$ and pKD46-$grpE_m$, the expression cassette of $grpE_m$ on pKD46-$grpE_m$ was chemically synthesized with synonymously mutated *grpE* whose expression was under the control of the promoter from chloramphenicol acetyltransferase gene Cat and the terminator from *rnpB* to make sure that $grpE_k$ fragments contained no similar sequences with pKD46-$grpE_m$. After strain conjugation and the first round of single crossover, the positive clones could grow on/in media amending with Amp and Cm, and was sensitive to sucrose due to the expression of *sacB1* from pRE112. Twenty positive clones were confirmed by PCR (Fig. 1a and 1c) and then replica-plated onto LB agar plates. After second round of single exchange, two kinds of recombinants formed: (i) the positive mutants in which the coding sequences of *grpE* were knocked out; (ii) and the reverse mutants in which the *grpE* alleles were back to wild type (Fig. 1b). The positive mutants were screened by PCR amplification (Fig. 1d) and confirmed by DNA sequencing. One of the positive mutants was chosen and assigned as PGH-0 which was used for further study. It should be emphasized that PGH-0 harbors the helper plasmid pKD46-$grpE_m$ and should be grown preferably at lower temperatures (30℃ or lowers are proposed).

grpE relocation on plasmid enhance the tolerance of host to toxic chemicals

To examine the effects of *grpE* relocation on plasmid, the growth profiles of PGH-0 and BL21(DE3) were compared by adding PG solution (in DMSO) to the liquid media at final concentration of 2.5 g/L of PG and 1% (v/v) of DMSO. It was found that higher stationary OD_{600} values were obtained in PGH-0 compared with BL21(DE3) under PG and/or DMSO stresses (Fig. 2), which suggested that PGH-0 carrying multicopy of pKD46-grpEm exhibited increased tolerance to PG and DMSO.

Bacterial PG synthesis was enhanced by PAS

In our previous work, PG biosynthesis was achieved in *E. coli* BL(DE3) harboring two plasmids, pET-phlDmarA and pA-accADBC, in which two kinds of antibiotics (Kan and Cm) must be added to the media for plasmid maintenance during fermentation (Cao et al. 2011). In this study, the functional *phlD-marA* fragments on pET-phlDmarA were PCR amplified and inserted into pA-accADBC to generate pPGN-1, which was transformed into *E. coli* BL21(DE3) to form PGN-1. Then, the *grpE* expression cassette ($grpE_e$) and the *birA* gene were sequentially inserted into pPGN-1 to generate pPGNE-1 and pPGNE-2, which were respectively transformed into *E. coli* PGH-0 to generate PGNE-1 and PGNE-2. PGNE-1 and PGNE-2 successfully lost the helper plasmid pKD46-$grpE_m$ after be growing at 42℃ for 14 h (Fig. S2). All the strains were grown in the mineral salts broth and were induced by 0.2 mM IPTG for 18 hours.

PGNE-1 and PGNE-2 grow faster and got higher stationary OD_{600} and PG titer than PGN-1 whether with or without Cm stress, and even PGNE-2 displayed the best growth profile and PG biosynthesis (Fig. 3). In terms of the same strain under different Cm stress, all the strains grew faster without Cm stress than those with Cm. Concerning with PG production, PG was produced during exponential phase. The time courses of PG production of these three strains were similar with those of cell growth. PGNE-2 got the highest PG titer, reaching 1.78 g/L PG without Cm stress. Whereas, the PGN-1 with Cm produced 0.75 g/L PG. An increase of 137.3% was achieved. PGNE-1 without Cm stress got a PG titer of 1.26 g/L, higher than that of PGN-1 with Cm stress.

Discussion

With exhaustion of fossil sources, microbial manufacture of important chemicals, biofuels, and biomaterials provides a promising sustainable avenue. Biotechnical processes often be conducted in plasmid-based expression systems which often encounters plasmid instability despite selection stresses were always utilized. To reduce the impacts of plasmid instability, several PASs have been developed. Nevertheless, a few drawbacks still need to overcome. In this study, a novel PAS using an essential gene *grpE* without downstream metabolic products as selection marker was developed. This PAS contains two components: that is an expression host with chromatinic essential gene disrupted and expression plasmids carrying the corresponding selection marker.

The genome of *E. coli* contains more than 4200 genes, about 300 of which are essential genes (Baba et al. 2006). To choose an essential gene for construction of the PAS, two major points were taken into consideration: (i) the final product of this gene can't be easily transferred to other cells, even the host cells are lysed and (ii) overexpression of such a gene would not defect the growth of the host and had better protect the host cells from the toxicity of the metabolites. Based on these considerations, *grpE* encoding a cochaperone without catalytic synthesis activity to produce downstream chemicals was selected. GrpE plays the role of nucleotide exchange factor in the DnaK-DnaJ-GrpE chaperone system, a family of heat-shock proteins, which assists in various cellular processes (Liberek et al. 1991). The innate *grpE* was a low-level expressed gene. When it was relocated on multicopy plasmid under the control of weak Cat promoter, it might be slightly overexpressed. It can be concluded that relatively low-level overexpression of *grpE* under weak promoters can increase tolerance to toxic PG and probably improved the productivity of metabolic engineered *E. coli*.

Generally, most of naturally occurring and artificially constructed plasmids merely provide advantages to the hosts under specific environmental conditions such as conferring resistance to antibiotics or utilizing certain substances but rarely concern viability or survival of the host cells. Any plasmids, especially for those artificially constructed plasmids to complete heterologous metabolic pathway, may inflict an extra metabolic burden to the hosts in comparison to plasmid-free cells which always grow faster, reducing the proportion of product yielding cells and leading to losses of final product recovery (Zielenkiewicz and Ceglowski 2001). To ensure the plasmids distribute equally in the two descendant cells, several mechanisms have been evolved: (i) site-specific recombination systems involved in random distribution of high-copy number plasmids to the descendants, (ii) active partition process which precisely distributes low and median copy number plasmids to daughter cells and (iii) PASs that selectively kill the plasmid-free daughter cells (Zielenkiewicz and Ceglowski 2001). Antibiotic-free biosynthesizing processes using PASs are cost-friendly for industrial fermentation. Recently, several PASs have been developed (Kroll et al. 2011; Sánchez-Pascuala et al. 2017; Zhao et al. 2019). Most of them used genes encoding essential enzymes which catalyzed metabolism or catabolism of chemicals which were barely existed in the media. However, these systems are still not applicable in the industrial fermentation of toxic chemicals. The novel PAS developed here increases the tolerance of host to environmental stresses and is much faithfully beneficial for production of toxic chemicals such as PG.

PG is one of the structurally simplest polyketides and is an important platform chemical, which was synthesized by chemical methods traditionally. In 2005, Achkar et al. published the first report of heterologously microbial synthesis of PG by solo expression of *phlD* in *E. coli* (Achkar et al. 2005). PhlD catalyzes three malonyl-CoA to form one PG. From then on, many efforts have been tried to increase the yield and productivity of PG in this bacterium. Overexpression of acetyl-CoA carboxylase complex encoded by *accADBC* to increase malonyl-CoA supply efficiently increased PG titer (Cao et al. 2011). Rao et al. increased the activity of PhlD by directed evolution of phloroglucinol synthase PhlD and improved the stability for practical PG production (Rao et al. 2013). Recently, PG biosynthesis was established in *Arabidopsis thaliana* (Abdel-Ghany et al. 2016), leading to accumulation of PG and PG-glucoside in the

transgenic lines. However, hitherto the titers of PG still hardly meet the industrial application. This is mainly caused by the instability of the plasmids and the inherent toxicity of PG to the host.

Toxicity symptoms and growth reduction often be associated with the PG producing hosts. Enhancing the tolerance of the hosts to PG as well as other valuable metabolites is a promising strategy for higher titers. Overexpression of MarA, a DNA-binding transcriptional dual regulator involved in multiple antibiotic resistance, and GroESL, another molecular chaperone system, improved the *E. coli* tolerance to PG and the production as well (Cao et al. 2011; Zhang et al. 2017). Besides metabolic engineering, fermenting optimization are often be used to increase the production. Nutrient composition and fermentation parameters such as feeding strategies, temperature, pH, dissolved oxygen, and other variables significantly affect the cell metabolism and metabolite production (Kumar and Shimizu, 2011). Continuous or semi-continuous fermentation has been utilized for decades. In situ extraction has been proved to be a very effective method, which can abate the toxicity of the metabolites, prolong the production time, and improve the titer (Yang et al. 2017). Nevertheless, strain improvements are still the base of microbial synthesis. To increase the stability for PG biosynthesis, we disrupted an essential gene *grpE* on the chromatin and relocated it on plasmid to generate the first PG producing strain PGNE-1, in which the PG titer was increased to 1.26 g/L from 0.75 g/L (produced by PGN-1 under antibiotic stress) in shaking flask fermentation. That was further increased to 1.78 g/L after overexpression of *birA*. BirA is a bifunctional protein that acts as regulator of the biotin operon and possesses biotin ligase activity to AccB, β subunit of acetyl-CoA carboxylase complex. Biotin serves as the prosthetic group covalently attached to a specific lysyl residue of AccB. BirA-mediated biotinylation of AccB plays essential role for the acetyl-CoA carboxylase complex (Blanchard et al. 1999). The increase of PG titer in *E. coli* PGNE-2 may result from the improved activity of acetyl-CoA carboxylase complex by the increased biotinylating level of this enzymatic complex by overexpressed active BirA.

In summary, a novel PAS using an essential gene *grpE* as selection marker was constructed, which could eliminate those plasmid-free cell to increase the hoard-working portion. In this system, the selection marker was overexpressed at a low level, which could improve the cell growth and increase tolerance of the host to toxic metabolites. When this PAS was applied in PG microbial synthesis, it significantly increased the PG titers from 0.75 g/L to 1.78 g/L in shaking flask fermentation, an increasing of 137.3%. These results indicate that biosynthesis of toxic PG using *grpE* based PAS in *E. coli* is a versatile strategy, which may be expanded to more biobased chemicals.

Declarations

Availability of data and material All data generated or analyzed during this study are included in this published article.

Code availability Not applicable.

Authors' contributions HZ conceived of the study and designed components of the research. JW proposed the idea, performed the experiments, and prepared the manuscript draft. XM and YC analyzed

and interpreted the data. All authors read and approved the final manuscript.

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Consent to participate Not applicable.

Consent for publication All authors declare that this article has not been published elsewhere and agree to publish it in this journal.

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References

Abdel-Ghany SE, Day I, Heuberger AL, Broeckling CD, Reddy ASN (2016) Production of phloroglucinol, a platform chemical, in *Arabidopsis* using a bacterial gene. Sci Rep-Uk 6(1):38483 doi:10.1038/srep38483

Achkar J, Xian M, Zhao HM, Frost JW (2005) Biosynthesis of phloroglucinol. J Am Chem Soc 127(15):5332-5333 doi:10.1021/ja042340g

Ang D, Georgopoulos C (1989) The heat-shock-regulated *grpE* gene of *Escherichia coli* is required for bacterial growth at all temperatures but is dispensable in certain mutant backgrounds. J Bacteriol 171(5):2748-2755 doi:10.1128/jb.171.5.2748-2755.1989

Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, Mori H (2006) Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol 2:2006.0008 doi:10.1038/msb4100050

Blanchard CZ, Chapman-Smith A, Wallace JC, Waldrop GL (1999) The biotin domain peptide from the biotin carboxyl carrier protein of *Escherichia coli* acetyl-CoA carboxylase causes a marked increase in the catalytic efficiency of biotin carboxylase and carboxyltransferase relative to free biotin. J Biol Chem 274(45):31767-9 doi:10.1074/jbc.274.45.31767

Cahen R (1962) Phloroglucinol: a pharmacological reevaluation. I. Smooth muscle relaxing properties. Arch Int Pharmacodyn Ther 138:311-9

Cao Y, Jiang X, Zhang R, Xian M (2011) Improved phloroglucinol production by metabolically engineered *Escherichia coli*. Appl Microbiol Biotechnol 91(6):1545-52 doi:10.1007/s00253-011-3304-5

Cooper TF, Heinemann JA (2000) Postsegregational killing does not increase plasmid stability but acts to mediate the exclusion of competing plasmids. Proc Natl Acad Sci U S A 97(23):12643-8 doi:10.1073/pnas.220077897

Cranenburgh RM, Lewis KS, Hanak JA (2004) Effect of plasmid copy number and lac operator sequence on antibiotic-free plasmid selection by operator-repressor titration in *Escherichia coli*. J Mol Microbiol Biotechnol 7(4):197-203 doi:10.1159/000079828

Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A 97(12):6640-6645 doi:10.1073/pnas.120163297

De Gelder L, Ponciano JM, Joyce P, Top EM (2007) Stability of a promiscuous plasmid in different hosts: no guarantee for a long-term relationship. Microbiology 153(2):452-463 doi:10.1099/mic.0.2006/001784-0

Edwards RA, Keller LH, Schifferli DM (1998) Improved allelic exchange vectors and their use to analyze 987P fimbria gene expression. Gene 207(2):149-157 doi:10.1016/S0378-1119(97)00619-7

Gao Y, Liu C, Ding Y, Sun C, Zhang R, Xian M, Zhao G (2014) Development of genetically stable *Escherichia coli* strains for poly(3-hydroxypropionate) production. Plos One 9(5):e97845 doi:10.1371/journal.pone.0097845

Harrison CJ, Hayer-Hartl M, Di Liberto M, Hartl F, Kuriyan J (1997) Crystal structure of the nucleotide exchange factor GrpE bound to the ATPase domain of the molecular chaperone DnaK. Science 276(5311):431-5 doi:10.1126/science.276.5311.431

Hoffman DM, Fontes AT (2010) Density distributions in TATB prepared by various methods. Propell Explos Pyrot 35(1):15-23 doi:10.1002/prep.200800069

Kang KA, Lee KH, Chae S, Zhang R, Jung MS, Ham YM, Baik JS, Lee NH, Hyun JW (2006) Cytoprotective effect of phloroglucinol on oxidative stress induced cell damage via catalase activation. J Cell Biochem 97(3):609-20 doi:10.1002/jcb.20668

Kawano M, Aravind L, Storz G (2007) An antisense RNA controls synthesis of an SOS-induced toxin evolved from an antitoxin. Mol Microbiol 64(3):738-754 doi:10.1111/j.1365-2958.2007.05688.x

Kroll J, Klinter S, Schneider C, Voss I, Steinbüchel A (2010) Plasmid addiction systems: perspectives and applications in biotechnology. Microb Biotechnol 3(6):634-657 doi:10.1111/j.1751-7915.2010.00170.x

Kroll J, Klinter S, Steinbuechel A (2011) A novel plasmid addiction system for large-scale production of cyanophycin in *Escherichia coli* using mineral salts medium. Appl Microbiol Biotechnol 89(3):593-604

doi:10.1007/s00253-010-2899-2

Kroll J, Steinle A, Reichelt R, Ewering C, Steinbuchel A (2009) Establishment of a novel anabolism-based addiction system with an artificially introduced mevalonate pathway: complete stabilization of plasmids as universal application in white biotechnology. Metab Eng 11(3):168-77 doi:10.1016/j.ymben.2009.01.007

Lau BTC, Malkus P, Paulsson J (2013) New quantitative methods for measuring plasmid loss rates reveal unexpected stability. Plasmid 70(3):353-361 doi:10.1016/j.plasmid.2013.07.007

Li L, Liu XC, Wei KK, Lu YH, Jiang WH (2019) Synthetic biology approaches for chromosomal integration of genes and pathways in industrial microbial systems. Biotechnol Adv 37(5):730-745 doi:10.1016/j.biotechadv.2019.04.002

Liberek K, Marszalek J, Ang D, Georgopoulos C, Zylicz M (1991) *Escherichia coli* DnaJ and GrpE heat shock proteins jointly stimulate ATPase activity of DnaK. Proc Natl Acad Sci U S A 88(7):2874-2878 doi:10.1073/pnas.88.7.2874

Liu M, Yao L, Xian M, Ding YM, Liu HZ, Zhao G (2016) Deletion of *arcA* increased the production of acetyl-CoA-derived chemicals in recombinant *Escherichia coli*. Biotechnol Lett 38(1):97-101 doi:10.1007/s10529-015-1953-7

Manoil C, Beckwith J (1985) Tn*phoA*: a transposon probe for protein export signals. Proc Natl Acad Sci U S A 82(23):8129-8133 doi:10.1073/pnas.82.23.8129

Nowak-Thompson B, Gould SJ, Kraus J, Loper JE (1994) Production of 2,4-diacetylphloroglucinol by the biocontrol agent *Pseudomonas fluorescens* Pf-5. Can J Microbiol 40(12):1064-1066 doi:10.1139/m94-168

Ogura T, Hiraga S (1983) Mini-F plasmid genes that couple host cell division to plasmid proliferation. Proc Natl Acad Sci U S A 80(15):4784-4788 doi:10.1073/pnas.80.15.4784

Palazzotto E, Tong Y, Lee SY, Weber T (2019) Synthetic biology and metabolic engineering of actinomycetes for natural product discovery. Biotechnol Adv 37(6):107366 doi:10.1016/j.biotechadv.2019.03.005

Rao GD, Lee JK, Zhao HM (2013) Directed evolution of phloroglucinol synthase PhlD with increased stability for phloroglucinol production. Appl Microbiol Biot 97(13):5861-5867 doi:10.1007/s00253-013-4713-4

Roland K, Curtiss R, Sizemore D (1999) Construction and evaluation of a *Δcya Δcrp Salmonella typhimurium* strain expressing avian pathogenic *Escherichia coli* O78 LPS as a vaccine to prevent airsacculitis in chickens. Avian Dis 43(3):429-441 doi:Doi 10.2307/1592640

Sambrook JF, Russell DW (2001) Molecular Cloning: A Laboratory Manual (3-Volume Set). vol 1, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp 1.116-1.118

San Millan A, MacLean RC (2017) Fitness costs of plasmids: a limit to plasmid transmission. Microbiol Spectr 5(5):MTBP-0016-2017 doi:10.1128/microbiolspec.MTBP-0016-2017

Sánchez-Pascuala A, de Lorenzo V, Nikel PI (2017) Refactoring the Embden-Meyerhof-Parnas pathway as a whole of portable GlucoBricks for implantation of glycolytic modules in Gram-negative bacteria. ACS Synth Biol 6(5):793-805 doi:10.1021/acssynbio.6b00230

Siddiqui IA, Shahid Shaukat S (2003) Suppression of root-knot disease by *Pseudomonas fluorescens* CHA0 in tomato: importance of bacterial secondary metabolite, 2,4-diacetylpholoroglucinol. Soil Biology and Biochemistry 35(12):1615-1623 doi:10.1016/j.soilbio.2003.08.006

Singh IP, Sidana J, Bansal P, Foley WJ (2009) Phloroglucinol compounds of therapeutic interest: global patent and technology status. Expert Opin Ther Pat 19(6):847-866 doi:10.1517/13543770902916614

Singh IP, Sidana J, Bharate SB, Foley WJ (2010) Phloroglucinol compounds of natural origin: synthetic aspects. Nat Prod Rep 27(3):393-416 doi:10.1039/B914364P

Summers DK (1991) The kinetics of plasmid loss. Trends Biotechnol 9(1):273-278 doi:10.1016/0167-7799(91)90089-Z

Tsang J (2017) Bacterial plasmid addiction systems and their implications for antibiotic drug development. Postdoc J 5(5):3-9

Wang C, Liwei M, Park JB, Jeong SH, Wei G, Wang Y, Kim SW (2018) Microbial platform for terpenoid production: *Escherichia coli* and yeast. Front Microbiol 9:2460 doi:10.3389/fmicb.2018.02460

Wang J, Huang C, Guo K, Ma L, Meng X, Wang N, Huo Y-X (2020) Converting *Escherichia coli* MG1655 into a chemical overproducer through inactivating defense system against exogenous DNA. Synth Syst Biotechnol 5(4):333-342 doi 10.1016/j.synbio.2020.10.005

Wang L, Chen H, Zhang T, Zhang J, Yang L (2007) Synthesis, characterization, thermal and explosive properties of potassium salts of trinitrophloroglucinol. J Hazard Mater 147(1):576-580 doi:10.1016/j.jhazmat.2007.01.043

Westbrook AW, Miscevic D, Kilpatrick S, Bruder MR, Moo-Young M, Chou CP (2019) Strain engineering for microbial production of value-added chemicals and fuels from glycerol. Biotechnol Adv 37(4):538-568 doi:10.1016/j.biotechadv.2018.10.006

Yang F, Cao Y (2012) Biosynthesis of phloroglucinol compounds in microorganisms–review. Appl Microbiol Biotechnol 93(2):487-95 doi:10.1007/s00253-011-3712-6

Yang H, Li J, Du G, Liu L (2017) Chapter 6 - Microbial production and molecular engineering of industrial enzymes: challenges and strategies. In: Brahmachari G (ed) Biotechnology of Microbial Enzymes. Academic Press, pp 151-165

Zha W, Rubin-Pitel SB, Zhao H (2006) Characterization of the substrate specificity of PhlD, a type III polyketide synthase from *Pseudomonas fluorescens*. J Biol Chem 281(42):32036-32047 doi:10.1074/jbc.M606500200

Zha W, Rubin-Pitel SB, Zhao H (2008) Exploiting genetic diversity by directed evolution: molecular breeding of type III polyketide synthases improves productivity. Mol Biosyst 4(3):246-248 doi:10.1039/B717705D

Zhang RB, Cao YJ, Liu W, Xian M, Liu HZ (2017) Improving phloroglucinol tolerance and production in *Escherichia coli* by GroESL overexpression. Microb Cell Fact 16:227 doi:10.1186/s12934-017-0839-x

Zhao L, Hu XQ, Li Y, Wang Z, Wang XY (2019) Construction of a novel *Escherichia coli* expression system: relocation of *lpxA* from chromosome to a constitutive expression vector. Appl Microbiol Biotechnol 103(17):7177-7189 doi:10.1007/s00253-019-10013-y

Zielenkiewicz U, Ceglowski P (2001) Mechanisms of plasmid stable maintenance with special focus on plasmid addiction systems. Acta Biochim Pol 48(4):1003-1023

Tables

Table 1 Plasmids and *E. coli* strains used in this study

Plasmids/Strains	Relevant characteristics	References
Plasmids		
pEasy-Blunt	Cloning vector; pUC ori, Amp^R, Kan^R, *lacZ*α	TransGen
pEasyB-$grpE_k$	pEasy-Blunt carrying the *grpE* knockour cassette	This study
pEasyB-$grpE_e$	pEasy-Blunt carrying *grpE* expression cassette from *E. coli*	This study
pEasy-pmf	pEasy-Blunt carrying *phlD-marA* expression fragment	This study
pEasy-birA	pEasy-Blunt carrying *birA* gene	This study
pUC57S-$grpE_m$	Cloning plasmid carrying the mutated *grpE* expression cassette; pUC ori, Amp^R, *lacZ*α	GenScript
pRE112	Suicide plasmid; T RP4 ori, R6K λ ori, *sacB*, Cm^R	(Edwards et al., 1998)
pRE112-$grpE_k$	pRE112 carrying the *grpE* knockout cassette	This study
pKD46	Red recombinase plasmid; $R101^{ts}$ ori, *repA101*, *gam*, *bet*, *exo*, *araC*, Amp^R	(Datsenko & Wanner, 2000)
pKD46-$grpE_m$	Helper plasmid; pKD46 carrying the mutated *grpE* expression cassette	This study
pET-phlDmarA	pET-30a(+) carrying *phlD* from *P. fluorescens* Pf-5 and *marA* from *E. coli*, Kan^R	(Cao et al., 2011)
pA-accADBC	pACYCDuet-1 carrying *accADBC* from *E. coli*, Cm^R	(Cao et al., 2011)
pPGN-1	PG producing plasmid with pmf inserted into pA-accADBC via *Afl*II/*Not*I, Cm^R	This study
pPGNE-1	PG producing plasmid with *grpE* expression cassette inserted into pPGN-1 via *Pac*I/*Xho*I, Cm^R	This study
pPGNE-2	PG producing plasmid with *birA* inserted into pPGNE-1 via *Stu*I/*Not*I, Cm^R	This study
***E. coli* Strains**		
χ7213	Host for pRE112-$grpE_k$; *thi-1 thr-1 leuB6 glnV44 tonA21 lacY1 recA1 RP4-2-Tc*::*Mu λpir ΔasdA4 Δzhf-2*::Tn*10*	(Roland et al., 1999)
CC118	Gene cloning strain specially for pRE112 derived plasmids; *araD139 Δ(ara leu)7697 DlacX74 DphoA20 galK thi rpsE rpoB argE(Am) recA1* (*λpir*)	(Manoil & Beckwith, 1985)
DH5α	Gene cloning strain; F^- *endA1 glnV44 thi-1 recA1 relA1 gyrA96*	Invitrogen

	deoR nupG purB20 φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *hsdR17*() λ^-	
BL21(DE3)	Initial strain; F^- *ompT gal dcm lon* $hsdS_B$() λ(DE3)	Invitrogen
R-0	Recipient strain during conjugation; B21(DE3) harboring the helper plasmid pKD46-$grpE_m$	This study
D-0	Donor strain during conjugation; χ7213 harboring pRE112-$grpE_k$	This study
PGH-0	Host strain; BL21(DE3) with disrupted *grpE*, harboring the helper plasmid pKD46-$grpE_m$	This study
PGN-1	PG producing recombinant strain; BL21(DE3) harboring pPGN-1	This study
PGNE-1	PG producing recombinant strain; PGH-0 harboring pPGNE-1	This study
PGNE-2	PG producing recombinant strain; PGH-0 harboring pPGNE-2	This study

Figures

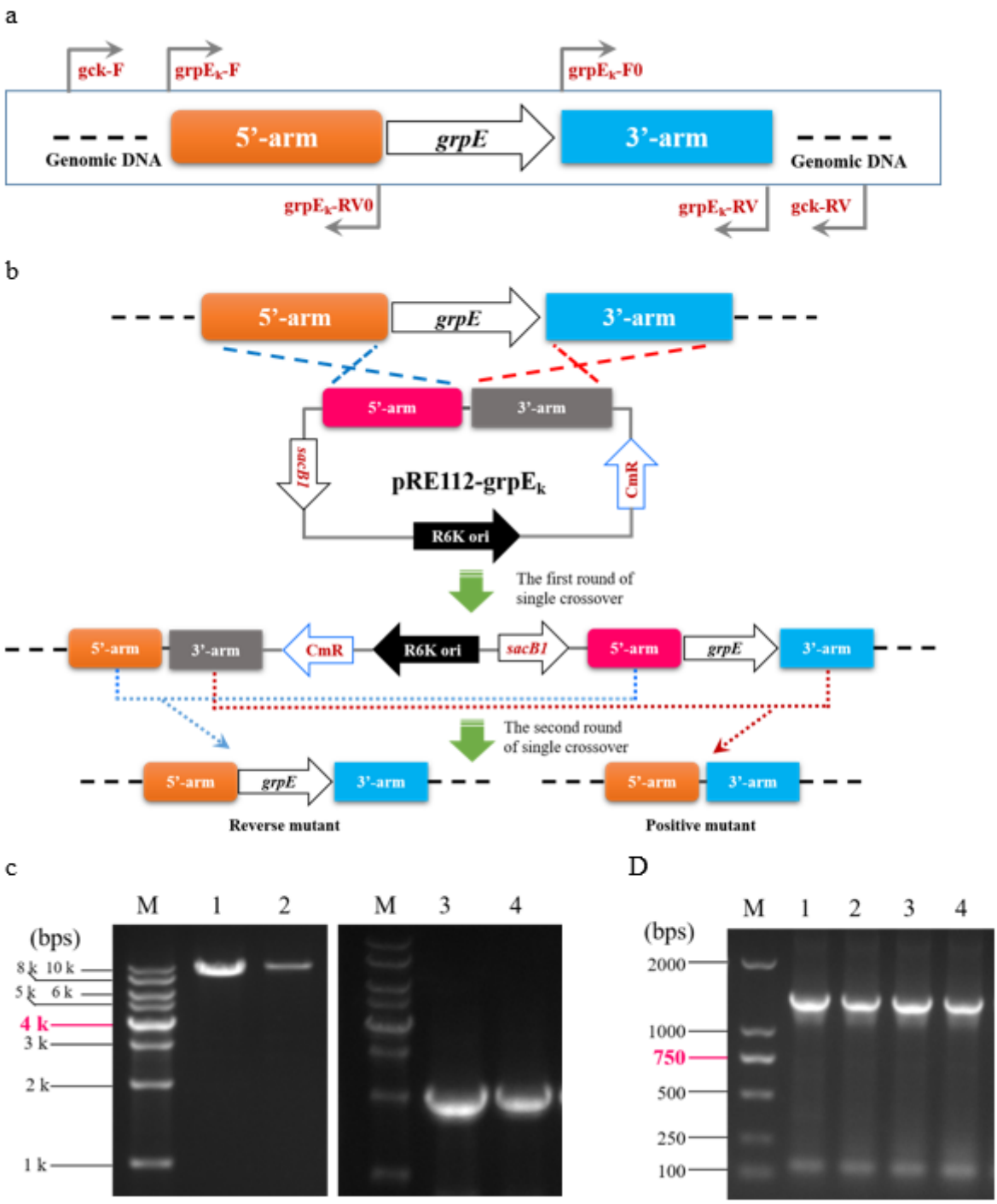


Figure 1

Construction of the ΔgrpE mutant of E. coli BL21(DE3). (a) Schematic representation of binding sites of different primers: gck-F/gck-RV are located 50 bps upstream and downstream of grpEk-F/grpEk-RV, respectively. (b) The schematic drawing illustrates the grpE knockout steps using allelic exchange method. The colors are used to identify different DNA fragments but not to indicate the different DNA resources. (c) Agarose electrophoresis photos of the PCR products from the recombinant cells after the first round of single crossover using gck-F/gck-RV as primers. The predicted length of the PCR products from positive clones is about 8300 bps. The results (Lanes 1 and 2) are consistent with the anticipation.

One hundred of clones were PCR examined. These two clones were tiny part of them. Lanes 3 and 4 were the controls using BL21(DE3) genomic DNA as template. (d) Agarose electrophoresis photos of the PCR products from the recombinant cells after the second round of single crossover using gck-F/gck-RV as primers. The PCR products were predicted to be 1300 bps long. The results were consistent with the prediction.

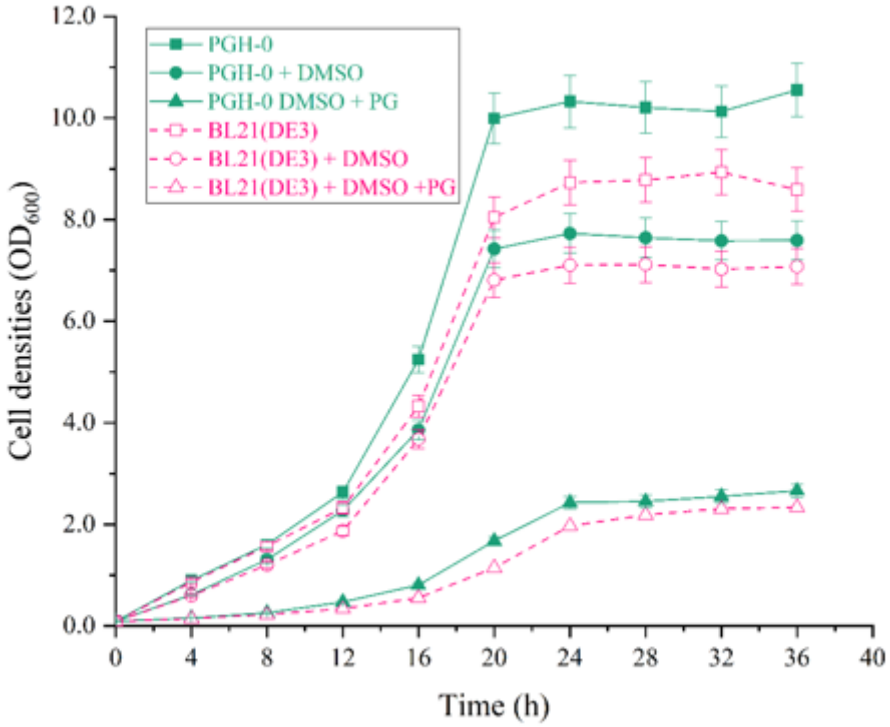
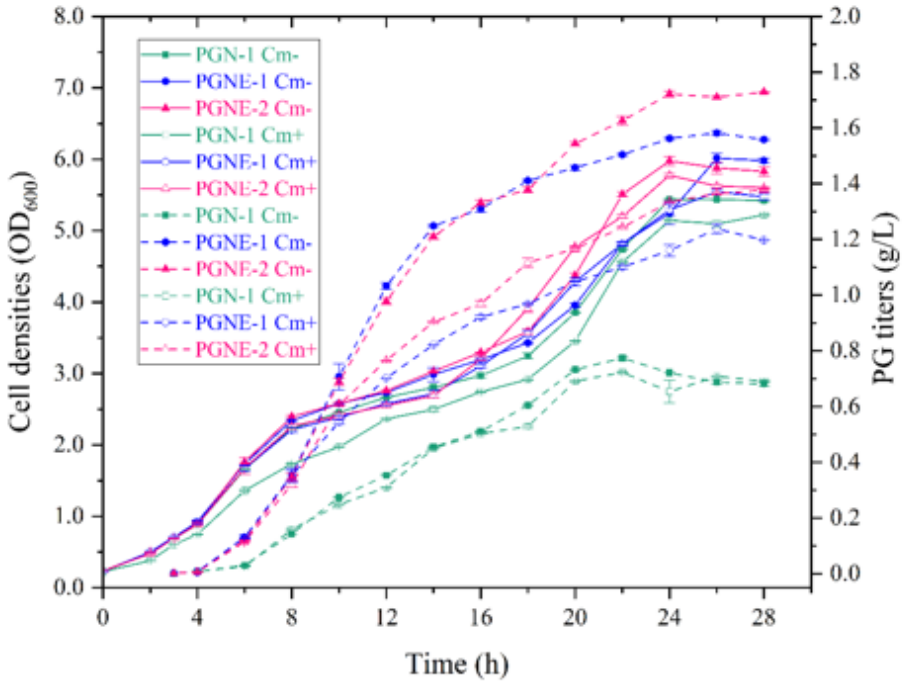


Figure 2

Time course of BL21(DE3) and PGH-0 growth under different toxic stresses. PGH-0 carrying multicopy grpE alleles relocated on plasmid obtained higher cell densities with or without toxic stress. Error bars indicate standard deviations (n = 3).

a



b

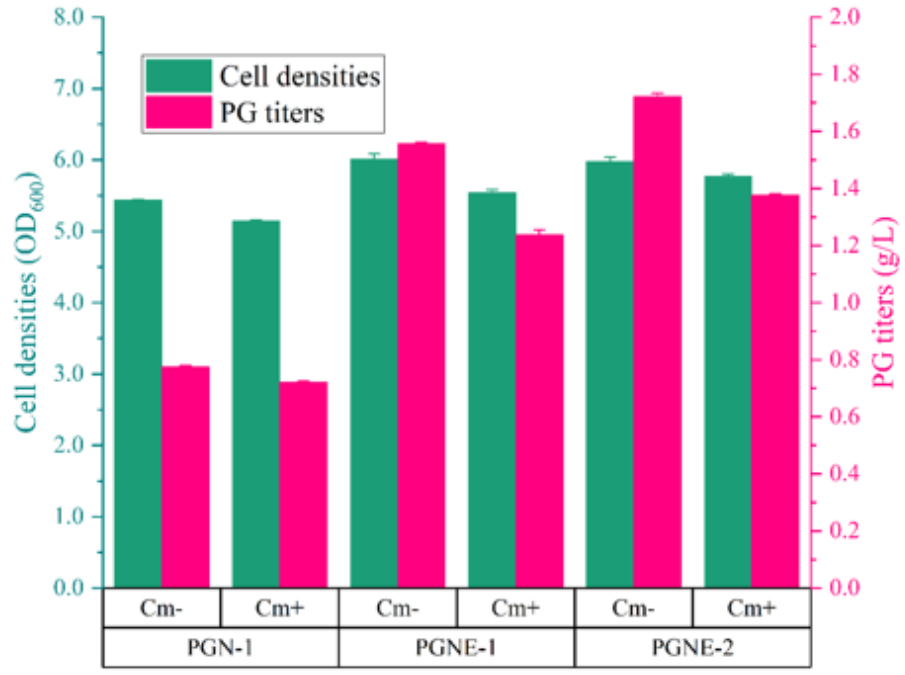


Figure 3

Microbial synthesis of PG was enhanced by the novel PAS. PGNE-2 and PGNE-1 grew faster and synthesized more PG than PGN-1 did. PGNE-2 and PGNE-1 produced 1.78 g/L and 1.26 g/L of PG without antibiotic stress respectively, while PGN-1 got an PG titer of 0.75 g/L under Cm stress. Error bars indicate standard deviations (n = 3).

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