Improved phloroglucinol production by metabolically engineered *Escherichia coli*

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**Abstract** Phloroglucinol is a valuable chemical which has been successfully produced by metabolically engineered *Escherichia coli*. However, the low productivity remains a bottleneck for large-scale application and cost-effective production. In the present work, we cloned the key biosynthetic gene, *phlD* (a type III polyketide synthase), into a bacterial expression vector to produce phloroglucinol in *E. coli* and developed different strategies to re-engineer the recombinant strain for robust synthesis of phloroglucinol. Overexpression of *E. coli marA* (multiple antibiotic resistance) gene enhanced phloroglucinol resistance and elevated phloroglucinol production to 0.27 g/g dry cell weight. Augmentation of the intracellular malonyl coenzyme A (malonyl-CoA) level through coordinated expression of four acetyl-CoA carboxylase (*ACCase*) subunits increased phloroglucinol production to around 0.27 g/g dry cell weight. Furthermore, the coexpression of *ACCase* and *marA* caused another marked improvement in phloroglucinol production 0.45 g/g dry cell weight, that is, 3.3-fold to the original strain. Under fed-batch conditions, this finally engineered strain accumulated phloroglucinol up to 3.8 g/L in the culture 12 h after induction, corresponding to a volumetric productivity of 0.32 g/L/h. This result was the highest phloroglucinol production to date and showed promising to make the bioprocess economically feasible.

**Keywords** Acetyl-CoA carboxylase · *marA* · Metabolically engineered *Escherichia coli* · *phlD* · Phloroglucinol

**Introduction**

Phloroglucinol is an important bulk chemical for multiple applications (Singh et al. 2009). Although the chemical synthetic processes of phloroglucinol have been established in the early twentieth century, the quickly rising petroleum prices and scarcity of fossil fuel supplies require the development of innovative synthesis techniques that are both cost-effective and energy saving. As a result, the use of biological processes for phloroglucinol and its derivatives production has been investigated by many former researchers (Validov et al. 2005; Weller et al. 2007). Phloroglucinol compounds are a major class of secondary metabolites that have been isolated from different natural sources (Singh and Bharate 2006). For instance, many species of *Pseudomonas* bacteria are capable of synthesizing phloroglucinol and 2,4-diacyethylphloroglucinol (Moynihan et al. 2009). Recently, a type III polyketide synthase cloned from *Pseudomonas fluorescens* (encoded by *phlD*) has been proved to be the key biosynthetic gene for phloroglucinol (Bangera and Thomashow 1999). In order to produce phloroglucinol in a common host, the PhlD enzyme was heterologously expressed in *Escherichia coli*, leading to the accumulation of phloroglucinol in cultures. Production of phloroglucinol under fermentor-controlled conditions using this recombinant strain reached 780 mg/L (Achkar et al. 2005).

However, the current productivity is still too low and not economically feasible for industrial applications. The fermentative production of phloroglucinol is limited by several different aspects. Phloroglucinol is the precursor of many antibiotics and its toxicity interferes further increase
of preliminary experiments showed that phloroglucinol significantly inhibited *E. coli* cell growth at a concentration of 0.5 g/L when added externally into a culture grown in mineral salts media. Improving the phloroglucinol resistance of *E. coli* is critical to achieve a higher titer. The multiple antibiotic resistance (*mar*) locus is a general resistance gene cluster and has shown to be involved in the resistance to many different antibiotics, disinfectants, and organic solvents (Randall and Woodward 2002). Overexpression of *marA* might improve the resistance of *E. coli* to phloroglucinol. In addition, biosynthesis of phloroglucinol requires a direct precursor, malonyl coenzyme A (malonyl-CoA). Wild-type *E. coli* only possesses a very low level of cellular malonyl-CoA for its natural metabolism, e.g., fatty acid anabolism. The insufficient supply of intracellular malonyl-CoA hampers high-level phloroglucinol production. Acetyl-CoA carboxylase (ACCase, EC 6.4.1.2), which catalyzes the irreversible carboxylation of acetyl-CoA, is the only producer of malonyl-CoA (Cronan and Waldrop 2002). Therefore, an obvious strategy to improve phloroglucinol production would be to increase ACCase level through episomal overexpression.

With the aim to enhance phloroglucinol production using recombinant *E. coli*, we reconstructed the metabolically engineered strains mainly from two points. First, a general resistance transcriptional activator gene, *marA* (multiple antibiotic resistance), was overexpressed to improve phloroglucinol tolerance. Second, the intracellular malonyl-CoA level was enhanced through the coordinated overexpression of four acetyl-CoA carboxylase (*ACCase*) subunits. The finally engineered strain showed a promising perspective for large-scale production of phloroglucinol.

**Materials and methods**

**Bacterial strains, plasmids, and culture media**

*E. coli* DH5α and BL21 (DE3) were used for all plasmid constructions and expression of recombinant proteins. The expression vectors pET30a and pACYCDuet-1 were purchased from Novagen (Madison, WI, USA). *E. coli* strains were cultivated in liquid Luria-Bertani (LB) media or on LB agar plates for DNA manipulation. For phloroglucinol production, recombinant strains were cultured using M9 minimal salts media supplemented with 1 mM MgSO₄ and 2% glucose as the carbon source. Fermentation media containing 0.3% (NH₄)₂SO₄, 0.1% citrate, 0.1% citrate sodium, 0.3% MgSO₄, 0.15% KH₂PO₄, 0.19% KCl, 0.00756% FeSO₄, and 2% initial glucose was used for fed-batch culture. Appropriate antibiotics (50 μg/ml of kanamycin or 34 μg/ml of chloramphenicol) were added to the culture media if necessary.

**Plasmids construction**

The recombinant plasmids and oligonucleotide primers used in this study are listed in Table 1. The *phiD* gene (GenBank accession no. EU554263) from *P. fluorescens* P5 (ATCC BAA-477) was cloned into pET30a vector between *NdeI* and *BamHI* sites, creating plasmid pET-phiD (Fig. 1a). The *marA* gene (GeneID: 6060688) from *E. coli* DH5α was firstly cloned into pACYCDuet-1 between the *Neol* and *BamHI* sites, resulting in plasmid pA-marA (Fig. 1b). Then PCR was performed using pA-marA as template to amplify the T7 promoter sequence along with the *marA* structural gene. The PCR product, T7marA was then cloned into pET-phiD between *EcoRI* and *SalI* sites, to create pET-phiDmarA (Fig. 1c). The four subunits of ACCase were PCR-amplified from *E. coli* DH5α genome. Then *accA* (GeneID: 6062185) and *accD* (GeneID: 6059083) were cloned into pACYCDuet-1, resulting in plasmids pA-accA and pA-accD, respectively. Subsequently, the T7accD fragment was amplified from plasmid pA-accD and cloned into pA-accA, creating pA-accAD. Finally, the accBC (GeneID: 6058890 and 6058863) fragment was cloned into pA-accAD to create plasmid pA-accADBC (Fig. 1d).

**Protein expression and gel electrophoresis analysis**

Single colonies of *E. coli* strain harboring different recombinant plasmids were used to inoculate liquid LB broth containing appropriate antibiotics and grown overnight. The colonized culture was diluted 1:100 in fresh LB media and incubated under the same conditions. When the optical density (OD) at 600 nm reached about 0.6 rel. AU, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM, and growth was continued for 3 h. The cells pelleted from 1 ml of culture were dissolved in 100 μl sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris–HCl pH 6.8, 2% SDS, 10% glycerol, 0.002% bromphenol blue, and 5% β-mercaptoethanol); heated to 100°C for 10 min; and then analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

**Shake-flask cultivation of the recombinant *E. coli* strains**

Shake-flask experiments were carried out in triplicate series of 500 ml Erlenmeyer flasks containing 50 ml of M9 minimal media. The culture broth was inoculated with *E. coli* strains harboring different recombinant plasmids and
incubated in a gyratory shaker incubator at 30°C and 200 rpm. When the OD600 of the culture reached about 0.6 rel. AU, IPTG was added to a final concentration of 0.1 mM to induce recombinant protein expression. Cell density, residual glucose, and phloroglucinol production were measured during the whole fermentation courses.

Fed-batch fermentation

Fed-batch cultures were carried out in a Biostat B plus MO5L fermentor (Sartorius Stedim Biotech GmbH, Germany) containing 3 L of growth media that was sterilized at 115°C for 30 min. Fifty milliliters of inoculum was

Table 1  Bacterial plasmids and oligonucleotide primers used in this study

| Plasmids | Description |
|----------|-------------|
| pET-phlD | pET30a harboring P. fluorescens Pf5 phlD |
| pA-marA | pET30a harboring E. coli DH5α marA |
| pET-phlDmarA | pET30a harboring P. fluorescens Pf5 phlD and E. coli DH5α marA |
| pA-accADBC | pACYCduet-1 harboring E. coli DH5α ACCase |

Oligonucleotide primers

| Primers | Sequence |
|---------|----------|
| phlD_F_NdeI | CATGCCATGGTAGATAAACGCGAATC |
| phlD_R_BamHI | ACGGTCGAGCTTTAGGTGTAATGATGATG |
| marA_F_Ncol | GAGAGATCCGAGCTTTAGGTGTAATGATG |
| marA_R_BamHI | CCGAATTTCTTATACGACTCAGTATAGGG |
| T7marA_F_EcoRI | TGGCGGTGGCAGCTTTAGGTGTAATGATG |
| T7marA_R_Sall | GAAATTCATAGTATTTATGATGATG |
| accA_F_BamHI | CGGGATCCGAGCTTTAGGTGTAATGATG |
| accA_R_SacI | ATGGAAATTCATAGTATTTATGATG |
| accBC_F_NdeI | CCGCTCGAGTTTCTTGAGACCCAGAG |
| accBC_R_NdeI | TGGCGGTGGCAGCTTTAGGTGTAATGATG |
| accD_F_BamHI | CGGGATCCGAGCTTTAGGTGTAATGATG |
| accD_R_SacI | ATGGAAATTCATAGTATTTATGATG |
| T7accD_F_Sall | GAAATTCATAGTATTTATGATGATG |
| T7accD_R_Sall | GAAATTCATAGTATTTATGATGATG |

Fig. 1  a–d Constructs for recombinant plasmids expressing phlD, marA, and ACCase
prepared by incubating the culture in shake-flasks overnight at 37°C. During the fermentation process, the pH was controlled at pH=7 via automated addition of ammonia. One percent of antifoam 204 (Sigma-Aldrich, USA) was added to prohibit foam development. The agitation was first set at 400 rpm and then associated with the dissolved oxygen (DO) to maintain a DO concentration of above 20% saturation. The bioreactor was first operated in a batch mode at 37°C until the initial glucose was nearly exhausted. Then fed-batch mode was commenced by feeding a solution containing 800 g/L of glucose at appropriate rates and the residual glucose was maintained less than 1 g/L. When the cells were grown to an OD600 of about 12 rel. AU, IPTG was added to the media at a concentration of 0.5 mM and the culture temperature was switched to 30°C. Fermentation parameters were the same as in shake-flask cultivation.

Analytical methods

Phloroglucinol resistance between different strains was compared by determining the minimal inhibition concentration (MIC) using the gradient-plate method (Hunt and Sandham 1969). E. coli cells after induction were streaked on LB plates containing 0.1 mM IPTG and different concentrations of phloroglucinol. Then the plates are incubated at 30°C for 36 h.

The intracellular malonyl-CoA level was analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) (Hayashi and Satoh 2006). The CoA pools of different E. coli strains were extracted from 100 ml of cultures with 0.25 M trichloroacetic acid using the method described by Wadler and Cronan (2007). LC-MS/MS was carried out using a LTQ XL-LC/MS system equipped with a linear ion trap mass analyzer (Thermo Fisher, USA). An X-Terra MS C18 reverse column (3.5 μm, 1.0×150 mm, Waters, USA) was used for HPLC separations. Methanol and 5 mM ammonium acetate were mixed for linear gradient elution at a flow rate of 0.1 ml/min. MS/MS scan conditions were as follows: the electrospray ion source was operated at 550°C and 3.5 kV in negative ion mode. The capillary voltage and temperature were 30 V and 200°C, respectively. Multiple reaction monitoring selected ions for acetyl-CoA and malonyl-CoA were 808.1/408.1 and 852.1/408.1, respectively.

The concentration of residual glucose in shake-flasks or fermentors was quantified by using an SBA-40D Biological Sensing Analyzer (Biology Institute of Shandong Academy of Sciences, China).

Phloroglucinol concentration in the fermentation supernatant was quantified using ion chromatography (IC). The experiments were performed on an ICS-3000 IC system (Dionex, USA) equipped with an IonPac AS11-HC column (4×250 mm, Dionex, USA) and an electrochemistry detector. A mixture of 100 mM NaOH and H2O was used for gradient elution at a flow rate of 0.5 ml/min. Suppression was achieved with an ASRS 300 (4 mm) anion suppressor (Dionex, USA) and the suppressor current was set to 100 mA.

Results

Cloning and expression of phlD, marA, and ACCase genes in E. coli

With the aim to express phlD, marA, and ACCase in E. coli, we cloned the coding region of these genes into pET30a and pACYCduet-1 expression vectors under the T7 promoter, respectively. The expression constructs were checked by colony PCR, restriction enzyme digestion, and DNA sequencing. To verify the expression levels of these recombinant proteins, E. coli BL21 (DE3) was transformed with the expression vectors including different genes and grown in LB liquid media followed by induction with 0.1 mM IPTG. Figure 2 shows the gel electrophoresis patterns of samples from different recombinant strains analyzed with Coomassie brilliant blue staining. In all cases, the recombinant proteins were clearly expressed and we noted distinct bands of the expected size in bacterial extracts of the recombinant strains when compared to the control strain.
Overexpression of marA enhances phloroglucinol resistance

The MICs of phloroglucinol were determined by the gradient-plate method. E. coli BL21 (DE3) harboring pACYCduet-1 and pA-marA was grown to an OD600 of 0.6 rel. AU and then induced with 0.1 mM IPTG for 2 h. Then the culture broth was inoculated on LB plates containing different concentrations of phloroglucinol. Overexpression of marA caused about 50% increase in resistance level to phloroglucinol in strain BL21 (DE3). The recombinant strain harboring pA-marA could endure a phloroglucinol concentration of 3.0 mg/ml while 2.1 mg/ml of phloroglucinol completely inhibited the control strain growth (Fig. 3).

Overexpression of ACCase enhances malonyl-CoA levels in E. coli

The intracellular malonyl-CoA concentration was determined using the LC-MS/MS analytical method described above. As expected, E. coli recombinant strain overexpressing ACCase also overproduced malonyl-CoA. A readily detectable level of malonyl-CoA, 0.30 nmol/mg dry cell weight (an OD600 of 1.0 rel. AU corresponds to 0.43 g dry cell weight per liter) was observed in the ACCase overexpression strain, about 3.6 times higher compared to the control strains persisting normal ACCase levels. This result was consistent with the reported value that was determined by an enzymatic method (Takamura and Nomura 1988).

Effect of marA and ACCase on phloroglucinol production

To test their abilities to produce phloroglucinol, all recombinant strains (E. coli overexpressing phlD, phlD&marA, phlD&ACCase, and phlD&marA&ACCase) were cultured in M9 salts media under shake-flask conditions. The different E. coli strains were grown to an OD600 of about 0.6 rel. AU and then induced for 12 h with 0.1 mM IPTG. The amounts of phloroglucinol accumulated in the culture media from different recombinant strains are shown in Fig. 4. When E. coli native ACCase was coexpressed with phlD, phloroglucinol concentration reached around 0.41 g/L after 12 h cultivation, about 1.8 times higher compared to the control strain only expressing phlD (0.23 g/L). Phloroglucinol productivity (gram phloroglucinol per gram dry weight) of this strain also increased from 0.14 to 0.27 g/gDW. In addition, this recombinant strain accumulated phloroglucinol much faster than the control strain (data not shown). Although strain phlD&marA could only give a slightly increase of the final phloroglucinol concentration, the productivity was greatly enhanced (0.27 g/gDW and 2-fold increase to the control strain). In the finally engineered strain coexpressing phlD, marA, and ACCase, the phloroglucinol contents reached 0.51 g/L and the productivity was 0.45 g/gDW, that is, 2.2-fold and 3.3-fold to the control strain. The molar yields of phloroglucinol on glucose in the strains of phlD, phlD&marA, phlD&ACCase, and phlD&marA&ACCase were 8.0%, 12.3%, 11.4%, and 16.0%, respectively. The finally engineered strain gave a 2-fold enhancement of phloroglucinol yield. Considering that glucose is required for cell growth and only 46% of glucose could be converted to acetyl-CoA under aerobic conditions (Zhao and Shimizu 2003), a reasonable yield of around 35% is expected. The current yield has the potential to be further improved.

Fed-batch culture of metabolically engineered E. coli strains

In order to test the suitability of the recombinant E. coli strains for an improved phloroglucinol production process, we established fed-batch fermentation based on the results obtained with flask cultures. The fermentations were carried out by using the fermentation media described above. E. coli strain harboring pET-phlD and pET-phlDmarA&pA-accADBC were further evaluated under fed-batch conditions. The residual glucose was maintained at a low level to avoid acetate accumulation throughout the fed-batch process. Growth, glucose utilization, and product accumulation were monitored over the course of the experiment. Figure 5 shows the time profiles for cell density and phloroglucinol concentrations during this experiment. For the recombinant strain only harboring pET-phlD, the OD600 and phloroglucinol concentrations obtained 12 h after being induced were 72.6 rel. AU and 1.8 g/L, respectively. When marA and ACCase were coexpressed with phlD gene, the recombinant strain grew much slower and cell growth ceased at an OD600 of around 40 rel. AU even though glucose was still
being consumed. However, the phloroglucinol production of this strain could reach 3.8 g/L (2.1-fold to the control strain) or 0.23 g/gDW (3.9-fold to the control strain) 12 h after induction, corresponding to a volumetric productivity of 0.32 g/L/h. This is the highest reported production of phloroglucinol by microbial fermentation. For both of the two recombinant strains, phloroglucinol accumulation hardly increased after 12 h cultivation.

Discussion

Phloroglucinol is an important pharmaceutical intermediate and can serve as a low toxic smooth muscle relaxant as well as being applied in the leather and chemical industries (Frost 2007). The current price of this valuable chemical on the international market has reached approximately 70 US $/kg. In biological organisms, phloroglucinol is a prototype of a class of complex natural products called polyketides. These biomolecules are biosynthesized from simple building block including acetyl-CoA, malonyl-CoA, and propionyl-CoA through the action of polyketide synthase (Pfeifer et al. 2001). An important plant-growth-stimulating bacteria, P. fluorescens, is able to produce 2,4-diacetylphloroglucinol (2,4-DAPG), a derivative of phloroglucinol (Shanahan et al. 1992; Slininger and Shea-Andersh 2005). The gene cluster (phl) involved in 2,4-DAPG biosynthesis has been comprehensively studied by many former researchers and the key biosynthetic gene for phloroglucinol, phlD, was demonstrated to be a type III polyketide synthase (Mavrodi et al. 2001; De La Fuente et al. 2006). PhlD enzyme catalyzes the synthesis of phloroglucinol from three molecules of malonyl-CoA (Zha et al. 2006).
is, *E. coli*, leads to accumulation of phloroglucinol in the cultures, providing new opportunities to produce this chemical through fermentation engineering. Since PhlD enzyme uses malonyl-CoA as the sole substrate to synthesize phloroglucinol, it can be hypothesized that the low concentration of cellular malonyl-CoA in *E. coli* might be a bottleneck for phloroglucinol accumulation. An obvious strategy to improve cellular malonyl-CoA concentration would be to increase ACCase levels through epistemic overexpression. It has been reported that overexpression of the ACCases from *Corynebacterium glutamicum* (Miyahisa et al. 2005) and *Photorhabdus luminescens* (Leonard et al. 2007; Fowler et al. 2009) in *E. coli* could lead to increased heterologous production of flavonones, whose precursor is also malonyl-CoA. Zha et al. (2009) indicated when the *phlD* gene was coexpressed with the ACCase from *C. glutamicum* in *E. coli* BL21 (DE3), the engineered strain improved phloroglucinol concentration approximately 2-fold (1,110 mg/L) after 48 h of cultivation compared to the wild-type, while overexpression of *E. coli* native ACCase could not enhance phloroglucinol production at all. In contrast to their work, we found that the native ACCase also gave a good performance by using the inducible T7 promoter expression system. Under shake-flask conditions, 0.41 g/L of phloroglucinol was reached in 12 h of cultivation, which showed a better productivity than 1,110 mg/L in 48 h of cultivation as reported (Zha et al. 2009). Previous studies showed that overexpression of *E. coli* native ACCase increased the rate of fatty acid synthesis and the pool of malonyl-CoA (Davis et al. 2000). Given the fact that malonyl-CoA is the sole precursor for phloroglucinol, it was not difficult to infer the native ACCase overexpressed strain would synthesize phloroglucinol more efficiently.

Product tolerance is an important issue affecting the application of microbes for the production of many valuable chemicals (Willke and Vorlop 2004; Antoni et al. 2007). The more substantial strategy to enhance phloroglucinol production is to improve the resistance of *E. coli*, because high concentration of phloroglucinol obviously inhibits cell growth. The multiple antibiotic resistance (mar) locus in *E. coli* consists of two divergently expressed operons (marC and marRAB). Both of them contribute to the resistance phenotype. The MarA protein, a member of the AraC family of transcriptional activators, mediates the response to multiple environmental stresses through the activation or repression in vivo of a large number of chromosomal genes, including *sodA*, *nfo*, *micF*, *inaA*, *fumC*, *zwf*, and *fpr* (Schneiders et al. 2004). Overexpression of marA from a plasmid is sufficient to confer multiple antibiotic resistances in *E. coli* (Gambino et al. 1993). The addition of antibiotics, aromatic weak acids, and a structurally diverse range of other compounds have all been shown to cause induction of mar regulon expression (Alekshun and Levy 1999). DNA microarray analysis indicated that MarA protein was strongly induced by externally added phloroglucinol to the culture media. In this study, we coexpressed the *marA* gene with *phlD*. The resulted strain produced higher final concentrations of phloroglucinol but grew much weaker than the original strain. This may be due to that MarA is a global transcription factor that activates a series of genes and thus influences the normal metabolism of the recombinant strain. Analyzing the downstream genes activated by MarA would be more crucial for improving phloroglucinol production.

The finally engineered strain coexpressing *marA* and ACCase together with *phlD* showed the highest production of phloroglucinol. Fed-batch fermentation of this strain accumulated phloroglucinol up to 3.8 g/L in the culture 12 h after induction. Compared with 780 mg/L of phloroglucinol under fermentor-controlled conditions for 48 h (Achkar et al. 2005), the titer and productivity of phloroglucinol were improved by both metabolic engineering and fermentation engineering strategies. The final strain gave a phloroglucinol yield of 16%. Despite the progress already obtained in recombinant *E. coli* for phloroglucinol production, there is still potential to increase titers, rates, and yields before industrial applications become economically feasible.

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