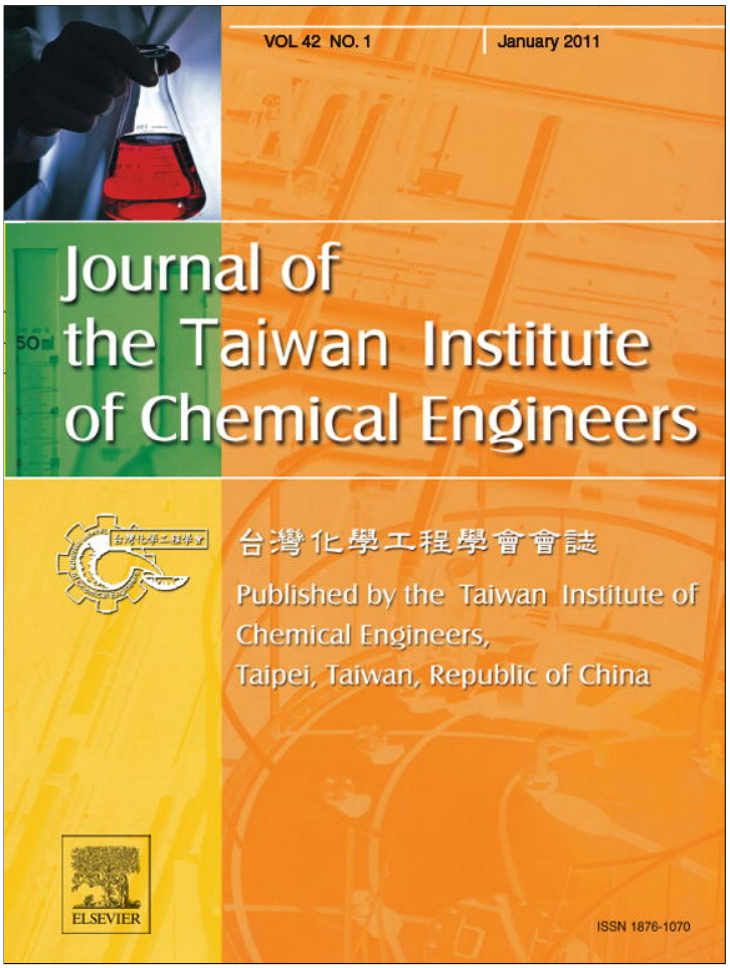

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Genomic engineering of *Escherichia coli* for production of intermediate metabolites in the aromatic pathway

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ABSTRACT

The approach by genetic engineering closely relies on the use of plasmids and is commonly afflicted by the potential problem of plasmid instability and safety concern. In this work, these issues were addressed by metabolic engineering of *Escherichia coli* for production of intermediate metabolites in aromatic pathway. By flux analysis, the pathway mode leading to maximal production yield was simplified with identification of key reaction steps. Making use of the developed methods for gene deletion and chromosomal insertion of genes, *E. coli* genome was manipulated free of antibiotic markers to redirect the pathway flux. Associated with the elimination of competing pathways, the rate-limiting steps were enhanced to supply precursor metabolites. As a consequence of *zwf* removal, it led the pathway flux to the destined path where quinic acid, gallic acid, and shikimic acid were accumulated. The result indicates the *zwf* as the critical node for redirection of carbon flux in this case. Overall, this study clearly illustrates the promise of the proposed methods for efficient manipulation of *E. coli* genome to alter its physiological trait.

1. Introduction

Escherichia coli has been frequently engineered for industrial applications. To have a new or improved trait, this strain is genetically manipulated by (i) elimination of undesirable genes, (ii) enhanced expression of key endogenous genes, or (iii) expression of heterologous genes with specific functions. Disruption of chromosomal genes in *E. coli* using the λ Red-based recombination system has proven very effective (Datsenko and Wanner, 2000). It starts with the use of polymerase chain reaction (PCR) to amplify a DNA fragment with two long extensions (36–50 base) homologous to the genomic gene of interest. Additionally, the core of this PCR DNA consists of an antibiotic-resistant gene flanked with two directly repeated FRT sites. By the act of λ-Red recombinases, the chromosomal gene was targeted with this synthetic DNA after being transformed and inactivated as a result of the crossover between the homologous regions of the PCR DNA and the chromosomal gene locus. Subsequently, the antibiotic-resistant marker of the inserted DNA can be removed upon acting on the flanking FRT sites by the Flp recombinase (FLP) provided from a helper plasmid.

For the ectopic expression of endogenous or heterologous genes, plasmids appear to be indispensable to attain multiple copies of target genes in host cells. However, it seems adverse to have manifold copies of plasmid-encoded genes for metabolic engineering of cells (Jones *et al.*, 2000). This is because the physiological stress caused to host cells by the redundant copy of DNAs can lead to segregational loss or internal rearrangements of plasmids (Peredelchuk and Bennett, 1997). In addition, the selective pressure is commonly applied to ensure the maintenance of plasmids in *E. coli*. As well recognized, the use of antibiotics is forbidden for some applications and the potential risk exits for the spread of the antibiotic-resistant trait to other microbes in nature (Julian *et al.*, 2001).

To tackle the above-mentioned problems, we have recently developed a series of integration plasmids for genomic insertion of native or foreign genes into the phage attachment site (Chiang *et al.*, 2008). Based on the CRIM plasmids with a conditional-replication origin (Haldimann and Wanner, 2001), these integration plasmids were constructed by bracketing the multiple cloning site (MCS) and a phage attachment (*attP*) locus (for phage λ, HK022, ϕ80, P21, and P22) with the FRT site. With the aid of a helper plasmid expressing the phage integrase (Int), these plasmids carrying the cargo genes can be integrated into their respective bacterial attachment (*attB*) site in single copy. As illustrated, the insertion efficiency of these integration plasmids was extremely high and integrants were highly stable as well. In

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addition, this approach is particularly attractive because it allows the site-specific insertion of DNAs into *E. coli* genome and later elimination of replicons (the self-replicating DNA fragments) and selective markers.

As proof of concept, in this study the metabolic pathway of *E. coli* was engineered with the developed method for directing the carbon flux to the committed pathway leading to aromatic amino acids. Associated with the removal of competing pathways by a newly explored method, the adopted strategy was to enhance the rate-limiting steps and to redirect the carbon flux to precursors. As a consequence, the pathway flux was channeled to the destination in the way as planned. A large amount of intermediate metabolites including quinic acid, gallic acid, and shikimic acid were found to accumulate in the cell. This result clearly indicates the promise of this method for efficient manipulation of *E. coli* genome to alter its physiological trait.

2. Materials and methods

2.1. Bacterial strains and culturing condition

Manipulation of DNAs was routinely conducted in *E. coli* strain DH5α(pir) (*deoR endA1 gyrA96 hsdR17 supE44 thi1 recA1 lacZM15 λpir*) whereas strain BL21(DE3) was engineered for the experiments. Recombinant strains were grown in shake flasks containing M9 mineral medium (Miller, 1972) supplemented with 2% glucose while their growth was monitored turbidimetrically at 550 nm (OD_{550}). Recombinant cells were grown for overnight and seeded to have an initial cell density of 0.1 at OD_{550}. The culture was maintained in an orbital shaker set at 200 rpm and 37 °C. Upon reaching 0.4 at OD_{550}, the cells were induced by adding 50 μM IPTG.

2.2. Plasmid construction

The plasmids and primers used in this study were listed in Table 1. The *tktA* and *talB* gene were synthesized from *E. coli* genome by PCR using primers RC0671-RC0672 and RC0673-RC0674, respectively. The PCR DNAs were subsequently digested either *Sac*I-*Xho*I or *Nde*I-*Sac*I and incorporated into pET-20bI (Wang *et al.*, 2004) to produce pET-TL. Meanwhile, the PCR DNAs comprising *aroF* and *ppsA* gene were produced with primers RC0677–RC0678 and RC0691–RC0692, respectively. The mutant form, $aroF^{FBR}$, was then created by site-directed mutagenesis of *aroF* with primer RC0675–RC0676 according to the previous report (Chao *et al.*, 2002). Followed by cleavage using either *Nde*I-*Sac*I or *Sac*I-*Xho*I, the resulting DNAs with $aroF^{FBR}$ and *ppsA* were ligated into pET-20bI to give pET-AS. Finally, the *glf* gene was amplified from *Zymomonas mobilis* genome using primer RC0693–RC0694. Pretreated with *Nde*I-*Xho*I, the DNA was spliced into pET-20bI to generate pET-Glf.

With the primer T7P-T7T, the gene(s) flanked by the T7 promoter and the T7 transcription terminator was produced by PCR from pET-TL, pET-AS, and pET-Glf. The resulting PCR DNAs were cloned into the *Pst*I-*Sma*I or *Sma*I site of pHK-Km, pΦ80-Km, and pP21-Km to produce pHK-TL, pΦ80-AS, and pP21-Glf, respectively.

2.3. Chromosomal insertion of genes

Strain BL21(DE3) and its derivatives were made competent for DNA transformation using calcium. Strains carrying a helper plasmid (*e.g.* pAH69, pAH121, or pAH123) expressing the phage Int were first cultured in LB medium at 30 °C and followed by exposure to 39 °C for 30 min when the cell density reached 0.3 at OD_{550}. The cells were then made competent and transformed with integration vectors containing genes to be inserted (pHK-TL, pΦ80-AS, or pP21-Glf). The resulting cells spread onto selective LB agar plates were tested for loss of the helper plasmid (conferring ampicillin sensitivity) and stable integration (conferring kanamycin resistance). To eliminate the region containing the selective marker and the replication origin, integrants bearing the inserted DNA were transformed with pCP20 expressing FLP (Datsenko and Wanner, 2000). Similarly, upon the thermal challenge by shifting 30–42 °C for 30 min, the resulting integrants were spread on non-selective LB medium at 39 °C for overnight and later examined for their susceptibility to antibiotics. Furthermore, the event of gene insertion and DNA deletion was verified by PCR.

2.4. Deletion of chromosomal genes

Strains carrying pK-HT (Herring and Blattner, 2004) were grown in LB medium (Miller, 1972) with 0.2% L-rhamnose to trigger the production of λ-Red recombinase at 30 °C. After reaching OD_{550} of 0.6, the cells were made electro-competent by concentrating 100-fold and washing two times with ice-cold 10% glycerol. For deletion of *ldhA*, two megaprimers were synthesized from *E. coli* genome using two primer sets, RC0681–RC0682 and RC0683–RC0684. Followed by overlapping PCR, the targeting DNA was generated by two megaprimers to prime the template plasmid pCm-ISc which carries the chloramphenicol-resistant gene blanked by two I-SceI sites. After purification, the targeting DNA (100 ng) was electroporated into competent cells and the electro-shocked cells were then incubated in SOC medium (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) at 37 °C for 2 h. The resulting cells were spread onto selective LB medium with the selection for chloramphenicol resistance at 37 °C. Meanwhile, two megaprimers were spliced by using PCR to produce the patch DNA. One integrant was made electro-competent and subsequently transformed with the patch DNA. The electro-shocked cells were then incubated in SOC medium with 0.01% tetracycline (for production of I-SceI) and 0.2% L-rhamnose at 37 °C for 2 h. Spread onto selective LB medium, strains were examined with the selection for chloramphenicol sensitivity (indicating loss of marker) and ampicillin sensitivity (loss of pK-HT). Similarly, the *ackA* gene was eliminated in the same way but using the primer set, RC0685–RC0686 and RC0687–RC0688.

2.5. Analytical methods

Glucose was measured by glucose assay kit (Sigma Co.). For analysis of quinic acid, gallic acid, and shikimic acid, high-pressure liquid chromatography (HPLC) was used using the LiChrosorb RP-18 column (YMC Tech.) with a mobile phase containing 6 mM H_3PO_4 (pH 2.1) pumped at 1 mL/min. The eluate was then monitored at 210 nm and at 70 °C. Alternatively, other fermentation products were analyzed using the YMC-Pack ODS-AQ column (YMC Tech.) with a mobile phase containing 20 mM H_3PO_4/NaH_2PO4 (pH 2.8). The eluate pumped at 0.7 mL/min was detected at 220 nm and at 30 °C.

3. Results

3.1. Analysis of the flux distribution in the model pathway

Before performing the genetic manipulation of cells, it is imperative to analyze the pathway leading to target products from which the theoretical yield is calculated and the potential limitations are identified in the carbon flux. Therefore, the flux analysis was carried out based on the constraint of pathway stoichiometry and mass balance. With schikimic acid in the

Table 1
Strains, plasmids, and primers used in this study.

	Relevant characteristics	Sources
Strain		
BL21(DE3)	ompT hsdS(r_B^- m_B^-) (DE3)	Novagen
BL21-1	As BL21(DE3) but *ldhA*$^-$ *ackA*$^-$	This study
BL21-F	As BL21-1 but *ptsG*$^-$ and inserted with *glf*, *talB-tktA*, and *aroF*^{FBR}-*ppsA*	This study
BL21-FZ	As BL21-F but *zwf*$^-$	This study
YS2231	*ptsG*::FRT-Km-FRT	Dr. C.-J. Chiang
YS2512	*zwf*::FRT-Km-FRT	Dr. C.-J. Chiang
Plasmid		
pHK-Km	Integration vector with $attP_{HK}$ and *kan*$^+$	This study
pΦ80-Km	Integration vector with $attP_{\phi}$ and *kan*$^+$	This study
pP21-Km	Integration vector with $attP_{P21}$ and *kan*$^+$	This study
pCP20	Helper plasmid expressing FLP with *bla*$^+$	Datsenko and Wanner (2000)
pK-HT	Helper plasmid expressing λ-Rad and I-SceI with *bla*$^+$	Herring and Blattner (2004)
pCm-ISc	Template vector with *ISceI-Cm-ISceI* cassette	Dr. C.J. Chiang
pΦ80-AS	As pΦ80-Km contains PT7:: *aroF*^{FBR}-*ppsA*	This study
pP21-Glf	As pP21-Km contains PT7::*glf*	This study
pHK-TL	As pHK-Km contains PT7::*talB-tktA*	This study
pAH69	Helper plasmid expressing phage HK022 Int with *bla*$^+$	Haldimann and Wanner (2001)
pAH121	Helper plasmid expressing phage P21 Int with *bla*$^+$	Haldimann and Wanner (2001)
pAH123	Helper plasmid expressing phage ϕ80 Int with *bla*$^+$	Haldimann and Wanner (2001)
Primer		
RC0681	TCTTATgAAACTCgCCgTTTATAg	
RC0682	ggTgAAAgTTggAACCTCTTACggAATTCACCggCACAgCgCAgg	
RC0683	TTAAACCAgTTCgTTCgggCAg	
RC0684	CgCgCgggTTTgTTACTgATAAAgAATTCgACgTgTATgAgAACgAAC	
RC0685	TTCCATgTCgAgTAAgTTAgTACTg	
RC0686	gAAgCAgCTCCAgCCTACACggAATTCACCggCACAgCgCAgg	
RC0687	gAAATCAggCAgTCAggCggCTC	
RC0688	ggTCgACggATCCCCggAATCAATTCgACgTgTATgAgAACgAAC	
RC0671	AgCTgAgCTCAggAggATACTATCATgTCCTCACgTAAAgAgCTTg	
RC0672	gAAACTCgAgTTACAgCAgTTCTTTTgCTTTC	
RC0673	AgCTCATATgACggACAAATTgACCTAgCAgC	
RC0674	CTAAgAgCTCTTACAgCAgATCgCCgATC	
RC0675	ggTgAAAgTTggAACCTCTTACggAATTCgATCATgAATggAACAAgg	
RC0676	CCTCAggCTgTCTATgTgTgACgAATTCAATCCgTggTTgCTCAAATC	
RC0677	TCgCCCATATgCAAAAAgACgCgCTg	
RC0678	TAATAgAgCTCTTAAgCCACgCgAgCCgTC	
RC0691	TTAgAgAgCTCAggATTgTTCgATgTCCAAC	
RC0692	ggATTCTCgAgTTATTTCTTCAgTTCAgCCAgg	
RC0693	TTACCATATgAgTTCTgAAAgTgTCAgg	
RC0694	AgCACTCgAgCTACTTCTgggAgCgCCAC	
T7P	AACgCTgCAggAgATCTCgATCTCgATCC	
T7T	ATCCggATCCAgTTCCTCCTTTCAg	

The sequences of primers homologous to plasmid pCm-ISc were underlined. Abbreviations: *kan*, kanamycin-resistant determinant; *bla*, ampicillin-resistant determinant; *Cm*, chloramphenicol-resistant determinant.

common aromatic pathway as the target, two reaction modes were worked out to obtain the maximal yield from glucose. As shown, the glycolytic flux is channeled into the pentose phosphate pathway (PPP) via either from G6P (Fig. 1a) or the condensation of F6P and G3P (Fig. 1b). They give the maximal molar yield of 67% and 85%, respectively. It should be noted that the calculated yield serves as a benchmark to work with but is not necessary to be consistent with the result that could be actually obtained.

In general, to ensure a high yield as suggested by the two reaction schemes (Fig. 1), PYR needs to be recycled back to PEP and the uptake of glucose should be bypassed from the phosphotransferase system (PTS) which results in PYR from PEP used for the transport of glucose into *E. coli* (Chao et al., 2003). Moreover, three key steps mediated by *aroF*, *talB*, and *tktA* were also identified. The former is responsible for the first committed step leading to the synthesis of aromatic amino acids. The latter two serve as the pivot nodes for shuttling the carbon flux in the PPP.

3.2. Elimination of waste pathways

As well recognized, *E. coli* undergoes a mixed acid fermentation, commonly producing acetate and lactate, irrespective of oxygen availability (El-Mansi and Holms, 1989). This will reduce the pyruvate pool and, consequently, lower the production yield. Therefore, competing for the utilization of pyruvate, the two reaction steps catalyzed by *ldhA* and *ackA* need to be eliminated.

To approach this goal, a scar-free method for chromosomal gene disruption was developed and the general protocol was outlined in Fig. 2. First, synthetic oligomers are designed to contain the priming sequences (Pm1, Pm2, Pm3, and Pm4) complementary to two ends of the chromosomal gene X and the extension sequences (H1 and H2) of 40–60 bases homologous to the template vector, pCm-ISc. With the primer set of Pm1–Pm2 and Pm3–Pm4, the two terminal regions of gene X are synthesized by PCR. Followed by overlapping PCR, the two synthetic DNA fragments (called megaprimers) are implemented to prime the template plasmid. As a result, it produces the targeting DNA consisting of the marker gene bracketed with I-SceI sites (*ISceI-Cm-ISceI*) and two homology extensions. After purification, the targeting DNA is electrotransformed into a host strain with the helper plasmid pK-HT which provides the λ-Red recombinases upon induction by L-rhamnose (Herring and Blattner, 2004). Integrants exhibiting resistance to the antibiotic are then examined for the inserted DNA using *in situ* PCR with primers Pm1–Pm4. Subsequently adding L-rhamnose and tetracycline, the λ-Red recombinases and I-SceI protein are triggered to produce from pK-HT in the integrant. The

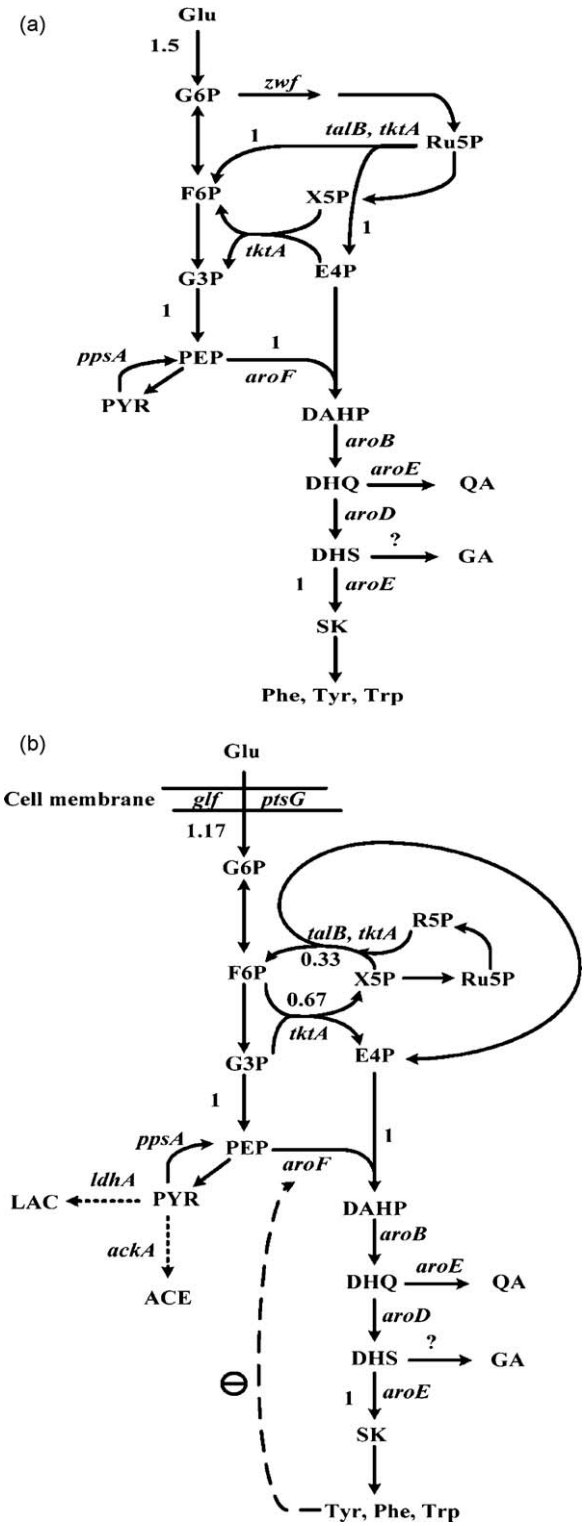

Fig. 1. Simplified reaction modes leading to the maximal production of the target metabolite. Flux analysis was carried out by assuming shikimic acid as the target. To achieve maximal production yield, pyruvate waste needs to be bypassed from the PTS and recycled to provide the precursor, phosphoenolpyruvate. Another precursor, erythose-4-phosphate, is supplied either from (a) the *zwf*-mediated pathway or (b) the *tktA*-catalyzed reaction step. The numerical values shown in the figures represent the relative molar flux based on the pathway stoichiometry. Abbreviations: Glu, glucose; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; G3P, glyceraldehyde 3-phosphate; PEP, phosphoenolpyruvate; PYR, pyruvate; LAC, lactate; ACE, acetate; 4P, erythose-4-phosphate; X5P, xylulose-5-phosphate; Ru5P, ribulose-5-phosphate; R5P, ribose-5-phosphate; DAHP, 3-deoxy-D-arabino-heptulsonate 7-phosphate; DHQ, 3-dehyroquinic acid; DHS, 3-dehydroshikimic acid; SK, shikimic acid; QA, quinic acid; GA, gallic acid; Phe, phenylalanine; Tyr, tyrosine; Trp, tryptophan. The activity of *aroF* subject to feed-back inhibition by Tyr was denoted by the dotted line.

marker in the inserted DNA is removed by the I-SceI digestion. In parallel, the two synthetic megaprimers are joined by PCR (called the patch DNA) and introduced into the host cell by electro-transformation. With the aid of the λ-Red recombinases, the gap left in the genome post the I-SceI cleavage is therefore repaired by the patch DNA fragment. The resulting transformants are screened for showing sensitivity to antibiotic (indicating the marker removal and the loss of pK-HT) after exposure to heat. Finally, the event of the gene disruption is verified by PCR using primers Pm1–Pm4.

On the basis of the protocol outlined above, both *ldhA* and *ackA* genes were then eliminated. Using pCm-ISc as the template, primers RC0681–RC0684 and RC0685–RC0688 (Table 1) were used to synthesize the gene fusion consisting of the *ISceI-Cm-ISceI* cassette sandwiched by the truncated parts of *ldhA* and *ackA*, respectively. After transformation of the linear DNA into *E. coli* strain BL21(DE3) bearing pK-HT, integrants showing resistance to chloramphenicol were selected and confirmed by PCR using primer RC0681–RC0684 (Fig. 3a) and RC0685–RC0688 (Fig. 3b). Later removal of the marker by the patch DNA was verified by PCR, and the resulting strain was designated BL21-1.

3.3. Enhanced activity of key metabolic steps

As revealed by the flux analysis (Fig. 1), at least 4 key reaction steps were identified and required to be enhanced, including *aroF*, *tktA*, *talB*, and *ppsA*. The enzymatic act of *ppsA* functions to redirect pyruvate back to PEP, a pivot step aimed at increasing the precursor pool and the production yield.

To up-regulate the expression of these genes, our developed method based on the integration vectors was employed for chromosomal insertion of target genes into phage attachment sites (Chiang *et al.*, 2008). As described, *tktA* and *talB* under control of the T7 promoter were constructed in an operon way and incorporated into integration vector pHK-Km. In a similar fashion, $aroF^{FBR}$ and *ppsA* were clustered into pΦ80-Km. The former gene is a mutant form of *aroF* and desensitized to the product (Tyr) inhibition (Weaver and Herrmann, 1990). First, the plasmid containing *tktA* and *talB* was transformed into strain BL21-1 carrying the helper plasmid pAH69 expressing phage HK Int. Upon temperature upshift, the *attP* (denoted by POP') and *attB* (denoted by BOB') sites were crossed over by the phage Int. Integrants with the insertion of DNA was then screened for exhibiting kanamycin resistance but ampicillin sensitivity (indicating the loss of the helper plasmid). Subsequently, the antibiotic-resistant determinant along with the replication origin was forced to remove by FLP expressed from pCP20. The resulting integrants susceptible to both kanamycin (loss of marker) and ampicillin (loss of pCP20) were examined for the inserted genes. Using PCR with specific primers (Table 1), it revealed the insertion of the gene cluster of *talB-tktA* (Fig. 4a). Afterwards, by a similar approach, the gene cluster of $aroF^{FBR}$-*ppsA* was integrated into the bacterial genome at the Φ80 attachment site and verified by PCR (Fig. 4b).

Moreover, to avoid generating pyruvate waste from glucose by the PTS, *ptsG* encoding the glucose transporter in *E. coli* was abolished by P1 transduction. This was done by preparing the infective phage particles from strain YS2231 (*ptsG*::FRT-Km-FRT) which was then transduced into the recipient cell for exhibiting resistance to kanamycin. The marker was subsequently removed by FLP using pCP20. The resulting strain was inserted with *glf* (the glucose facilitator from *Zymomonas mobilis*) based on the use of integration vector pP21-Km. By removal of the marker, the strain,

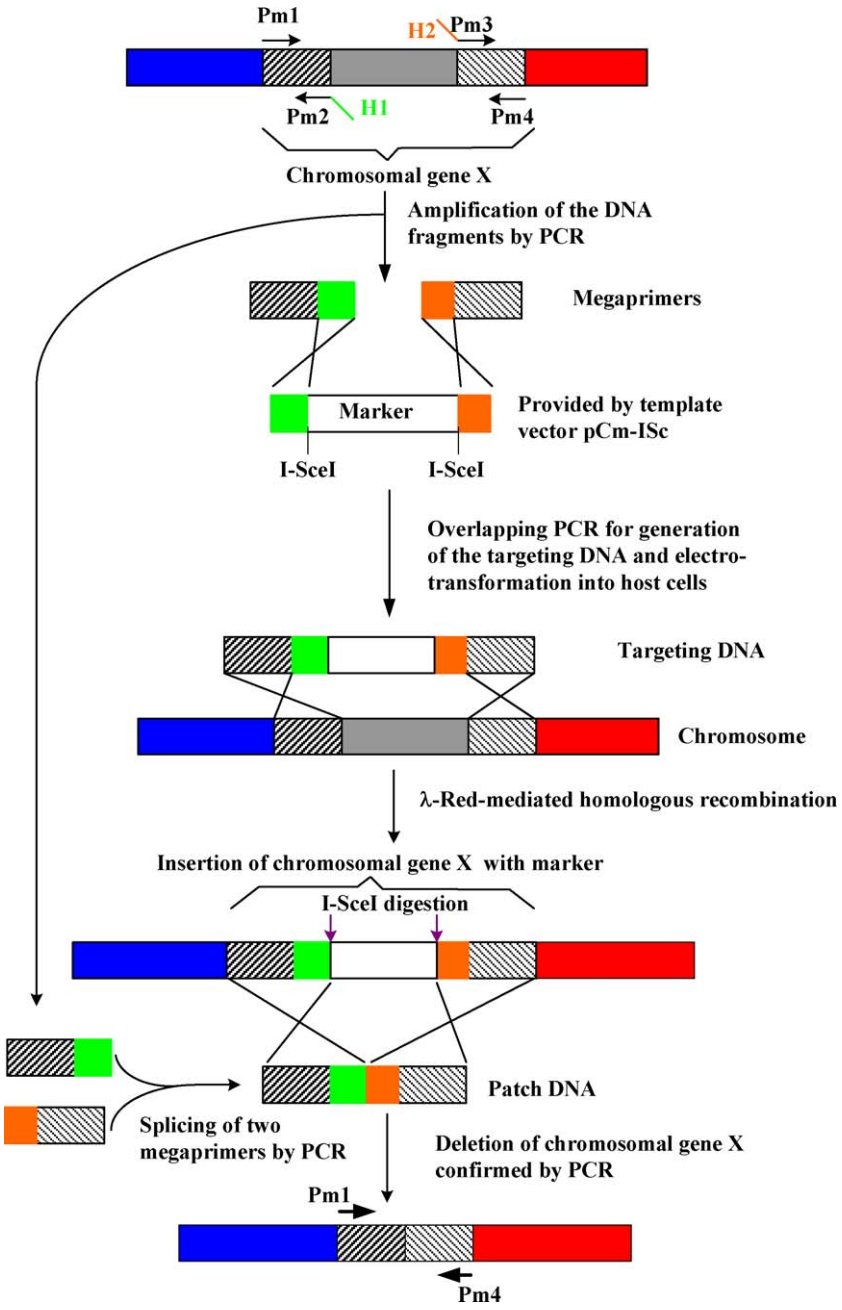

Fig. 2. Schematic illustration of the procedure for deletion of chromosomal genes. Refer to text for more details.

designated BL21-F, with the insertion of *glf* was confirmed by resuming the phenotype for glucose consumption.

3.4. Fermentative production of intermediate metabolites

To compare the two reaction modes depicted in Fig. 1, *zwf* gene was then disrupted by infecting strain BL21-F with the P1 phages preparing from YS2512 (*zwf*::FRT-Km-FRT). The transdutant showing kanamycin-resistant was examined for the presence of the marker gene by PCR. Later removal of the marker was processed in a similar way by FLP using pCP20, which gave the resulting strain BL21-FZ.

The performance of strain BL21-F and BL21-FZ was further investigated using shake-flask cultures. Upon induction by IPTG, the cell growth and fermentation products were measured along the time course. As shown in Fig. 5a, the final cell density for two experimental strains was lower than the control strain BL21-1. The result also showed that the control strain consumed glucose more rapidly (Fig. 5c), indicating that PTS is more efficient than *glf* for glucose transport. In particular, strain BL21-FZ grew relatively slow but was able to produce quinic acid, galic acid, and, to a less extent, shikimic acid (Fig. 5b). Strain BL21-F, however, produced only quinic acid whereas these metabolites were absent for the control strain (Fig. 5c). Interestingly, it was found that both the control strain and strain BL21-F were capable of accumulating pyruvate as the fermentation product (Fig. 5c).

4. Discussion

Strongly relying on the use of plasmids, genetic engineering appears to be a very powerful tool to manipulate cells for desired traits. However, it leads to the problem of plasmid burden which ultimately alters the host cell physiology and discourages the engineering purpose (Birnbaum and Bailey, 1991; Martinez-

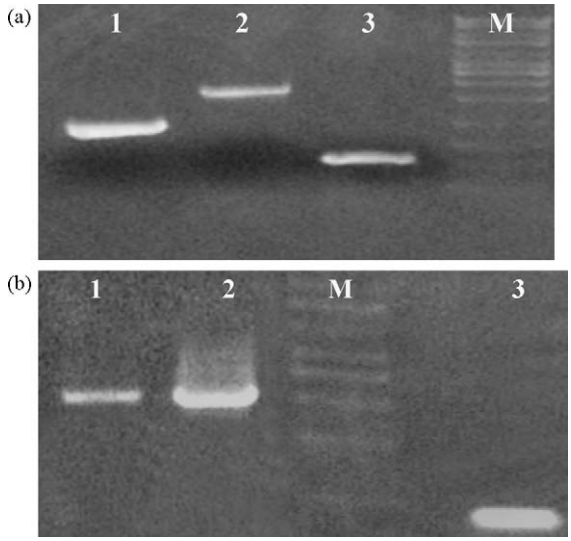

Fig. 3. Analysis of the gene deletion event and the marker removal by agarose gel electrophoresis. An integrant carrying *ldhA* and *ackA* deletion was verified by PCR with their specific primers. (a) Keys: M, the DNA marker; lane 1, the wild-type *ldhA* (0.9 kb); lane 2, *ldhA* with the insertion of *ISceI-Cm-ISceI* cassette (1.9 kb), lane 3, the remaining *ldhA* after the removal of the maker cassette (0.5 kb). (b) Keys: M, the DNA marker; lane 1, the wild-type *ackA* (2 kb); lane 2, *ackA* with the insertion of *ISceI-Cm-ISceI* cassette (2 kb), lane 3, the remaining *ackA* after the removal of the maker cassette (0.4 kb).

Morales *et al.*, 1999). In addition, the free release of plasmids containing the antibiotic marker and the replicon would certainly raise an environmental concern. In this study, these issues were addressed by using the developed methods for genomic gene deletion and the controlled expression of target genes completely

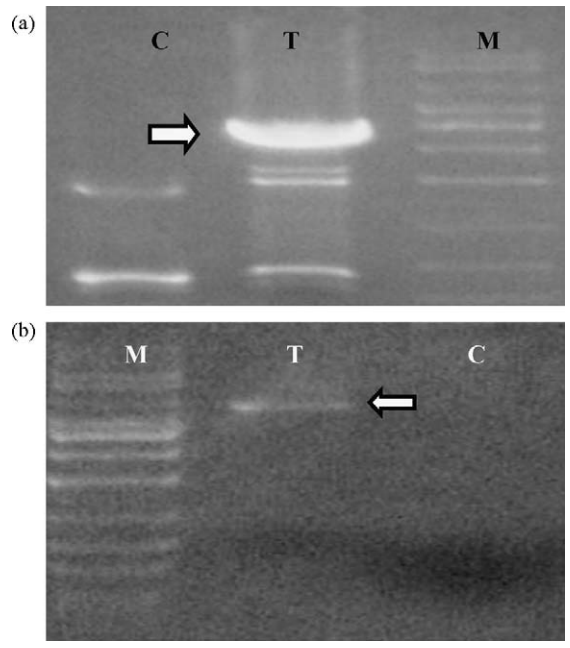

Fig. 4. Analysis of chromosomal insertion of gene clusters by agarose gel electrophoresis. The gene clusters were integrated into *E. coli* genome essentially following the protocol reported previously (Chiang *et al.*, 2008). The antibiotic-resistant marker was removed by FLP and selected by integrants showing sensitivity to the antibiotic. An integrant carrying the inserted the gene cluster was verified by PCR. (a) Keys: M, the DNA marker; C, the control strain without the gene cluster; T, the strain carrying the inserted gene cluster of *talB-tktA* (2.3 kb) as indicated by the arrow. (b) Keys: M, the DNA marker; C, the control strain without the gene cluster; T, the strain carrying the inserted gene cluster of *aroF*FBR-*ppsA* (3.8 kb) as indicated by the arrow.

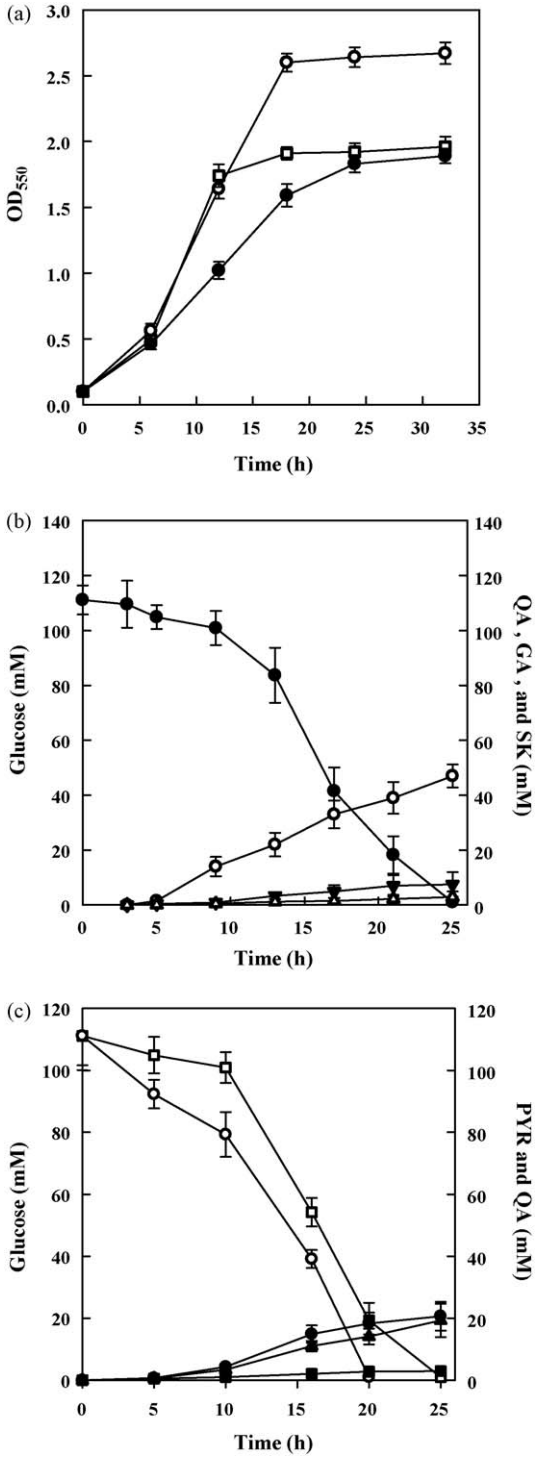

Fig. 5. As described in Section 2, strain BL21-1, BL21-F, and BL21-FZ were grown in shake flasks. Upon induction by IPTG, cells were sampled for determination of fermentation products and their growth was monitored along the time course. The experiments were duplicated. (a) Cell growth curve. Symbols: ○: strain BL21-1; □:strain BL21-F; ●: strain BL21-FZ. (b) Fermentation profile for strain BL21-FZ. Symbols: ●: glucose; ○: quinic acid; ▼: gallic acid; □: shikimic acid. (c) Fermentation profile for strain BL21-1 and BL21-F. Symbols: ○: glucose consumption for strain BL21-1; □: glucose consumption for strain BL21-F; ●: pyruvate production for strain BL21-1; ■: pyruvate production for strain BL21-F; ▲: quinic acid production for strain BL21-F. Refer to Fig. 1 legend for abbreviations.

free of replicons and markers. For gene knockout, the method lies in the use of I-SceI for the removal of the marker, leaving no scars in the genome (Fig. 2). This is unlike FLP which leaves one FRT site behind after recombination. Indeed, it is undesirable to have the extra FRT sites remained in the genome due to their interference with other FRT sites acted by FLP. Without residing in plasmids, genes of interest regulated by their unnatural promoter were all incorporated into the bacterial genome using the conditional-replication integration vectors. In contrast to the commonly used transposons (de Lorenzo and Timmis, 1994), genes are randomly integrated into the chromosomal locus, which creates the inserted DNAs in the form of replicons and transposable elements associated with selective markers. This will complicate subsequent constructions when cycling improvements are required. As illustrated here, the integration method allows the site-specific and repeated insertion of genes and offers many advantages for applications.

Aromatic compounds derived from the aromatic pathway are of great commercial importance. For instance, shikimic acid has been utilized as the starting material for the manufacture of Tamiflu, an orally effective antiinfluenza agent. It also provides a core scaffold for chemical synthesis of many compounds (Karpf and Trussardi, 2001). Additionally, pyrogallol derived from gallic acid serves as the precursor platform for the synthesis of other aromatic chemicals (Kambourakis *et al.*, 2000). In this work, an attempt was made to redirect the carbon flux from glucose into the aromatic pathway. This was approached first by flux analysis to simply the pathway mode leading to the maximal product yield (Fig. 1). Except *talB*, it picked out several key reaction steps identical with the finding reported previously, including *aroF*, *tktA*, *ppsA*, and *glf* (Jian *et al.*, 2002, 2003). Deprived of *ldhA* and *ackA*, the control strain BL21-1 was free of lactate and acetate waste produced which were replaced by pyruvate as the main fermentation product (Fig. 5c). The level of pyruvate was reduced and led to quinic acid when the pathway flux was rerouted in the strain BL21-F (Fig. 5b). By further abolishment of the *zwf*-mediated step, strain BL21-FZ became able to channel more carbon flux to quinic acid, gallic acid, and shikimic acid. This was compromised by lower production of biomass (Fig. 5a). In total, the production yield for three aromatic compounds could account for 47% molar conversion on glucose. Obviously, this result contradicts the prediction given by the working model, suggesting the existence of regulatory mechanisms that interfere with flux distribution. Moreover, no growth was taken into accounts and shikimic acid was considered as the end product when formulating the model (Fig. 1). Indeed, the downstream pathway leading to the synthesis of aromatic amino acids from shikimic acid was not blocked in this case. This is in contrast to the previous studies reporting to achieve the accumulation of upstream metabolites (Jian *et al.*, 2002, 2003). Nevertheless, the approach adopted here offers an advantage that the simplest medium can be used for cell culturing without supplement of aromatic amino acids. Most importantly, the working model predicted the *zwf*-mediated step as the critical node for increasing the production yield (Fig. 1), and this view was supported by the experimental approach as illustrated (Fig. 5b and c).

T7 promoter was chosen for utilization because of its strong strength and specific requirement for T7 RNA polymerase for activation (Studier and Moffatt, 1986). Acting like a regulon, an exclusive expression of the gene array could be achieved by IPTG (an inducer) which transmits a signal for production of T7 RNA polymerase. This should help to reduce the competition for the host RNA polymerase (Tabor and Richardson, 1985). Accordingly, it allows separation of the product production phase from the cell growth phase, and this is particularly useful for the case where the product is toxic to cells.

5. Conclusion

As with this successful illustration, it is conceived that the proposed methods can be extended to the other metabolite production where a clean production process is particularly concerned.

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References

Birnbaum, S. and J. E. Bailey, "Plasmid Presence Changes the Relative Levels of Many Host Cell Proteins and Ribosome Components in Recombinant *Escherichia coli*," *Biotechnol. Bioeng.*, **37**, 736 (1991).

Chao, Y. P., C. J. Chiang, Y. L. Wang, and Z. W. Wang, "Applicability of New Expression Vectors for Both Engineering Uses and Biological Studies," *Biotechnol. Prog.*, **19**, 1076 (2003).

Chao, Y. P., W. Law, P. Chen, and W. Hung, "High Production of Heterologous Proteins in *Escherichia coli* Using the Thermo-regulated T7 Expression System," *Appl. Microbiol. Biotechnol.*, **58**, 446 (2002).

Chiang, C. J., P. T. Chen, and Y. P. Chao, "Replicon-Rree and Markerless Methods for Genomic Insertion of DNAs in Phage Attachment Sites and Controlled Expression of Chromosomal Genes in *Escherichia coli*," *Biotechnol. Bioeng.*, **101**, 985 (2008).

Datsenko, K. A. and B. L. Wanner, "One-step Inactivation of Chromosomal Genes in *Escherichia coli* K-12 Using PCR Products," *Proc. Natl. Acad. Sci. U.S.A.*, **97**, 6640 (2000).

de Lorenzo, V. and K. N. Timmis, "Analysis and Construction of Stable Phenotypes in Gram-Negative Bacteria with Tn5- and Tn10-Derived Minitransposons," *Methods Enzymol.*, **235**, 386 (1994).

El-Mansi, E. and W. Holms, "Control of Carbon Flux to Acetate Excretion during Growth of *Escherichia coli* in Batch and Continuous Cultures," *J. Gen. Microbiol.*, **135**, 2875 (1989).

Haldimann, A. and B. L. Wanner, "Conditional—Replication, Integration, Excision, and Retrieval Plasmid-Host Systems for Gene Structure–Function Studies of Bacteria," *J. Bacteriol.*, **183**, 6384 (2001).

Herring, C. D. and F. R. Blattner, "Conditional Lethal Amber Mutations in Essential *Escherichia coli* Genes," *J. Bacteriol.*, **186**, 2673 (2004).

Jian, Y., K. M. Drath, L. Kai, and J. W. Frost, "Modulation of Phosphoenolpyruvate Synthase Expression Increases Shikimate Pathway Product Yields in *E. coli*," *Biotechnol. Prog.*, **18**, 1141 (2002).

Jian, Y., K. M. Drath, L. Kai, and J. W. Frost, "Altered Glucose Transport and Shikimate Pathway Product Yields in *E. coli*," *Biotechnol. Prog.*, **19**, 1450 (2003).

Jones, K. L., S. W. Kim, and J. D. Keasling, "Low-copy Plasmids Can Perform as Well as or Better than High-copy Plasmids for Metabolic Engineering of Bacteria," *Metab. Eng.*, **2**, 328 (2000).

Julian, A., J. Hanak, and R. M. Cranenburgh, "Antibiotic-free Plasmid Selection and Maintenance in Bacteria," In Merten, O.W., Mattanovich, D., Lang, C., Larsson, G., Neubauer, P., Porro, D., Postma, P., Teixeira, de M.J., Cole, J.A. (Eds.), Kluwer Academic, Dordrecht, Netherlands, p. 111 (2001).

Kambourakis, S., K. M. Draths, and J. W. Frost, "Synthesis of Gallic Acid and Pyroglol from Glucose: Replacing Natural Product Isolation with Microbial Catalysis," *J. Am. Chem. Soc.*, **122**, 9042 (2000).

Karpf, M. and R. Trussardi, "New, Azide-free Transformation of Epoxides into 1,2-Diamino Compounds: Synthesis of the Anti-Influenza Neuraminidase Inhibitor Osltamivir Phosphate (Tamiflu)," *J. Org. Chem.*, **66**, 2044 (2001).

Martinez-Morales, A., S. York, L. Yomano, V. Pineda, F. Davis, J. Shelton, and L. Ingram, "Biosynthetic Burden and Plasmid Burden Limit Expression of Chromosomally Integrated Heterologous Genes (*pdc*, *adhB*) in *Escherichia coli*," *Biotechnol. Prog.*, **15**, 891 (1999).

Miller, J. H., *Experiments in Molecular Genetics*, p. 431, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, (1972).

Peredelchuk, M. Y. and G. N. Bennett, "A Method for Construction of *E. coli* Strains with Multiple DNA Insertions in the Chromosome," *Gene*, **187**, 231 (1997).

Studier, F. W. and B. A. Moffatt, "Use of Bacteriophage T7 RNA Polymerase to Direct Selective High-Level Expression of Cloned Genes," *J. Mol. Biol.*, **189**, 113 (1986).

Tabor, S. and C. C. Richardson, "A Bacteriophage T7 RNA Polymerase/Promoter System for Controlled Exclusive Expression of Specific Genes," *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 1074 (1985).

Wang, Z. W., W. S. Law, and Y. P. Chao, "Improvement of the Thermoregulated T7 Expression System by Using the Heat-Sensitive *lacI*," *Biotechnol. Prog.*, **20**, 1352 (2004).

Weaver, L. M. and K. M. Herrmann, "Cloning of an *aroF* Allele Encoding a Tyrosine-Insensitive 3-Deoxy-D-Arabino-Heptulosonate 7-Phosphate Synthase," *J Bacteriol.*, **172**, 6581 (1990).