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Replicon-Free and Markerless Methods for Genomic Insertion of DNAs in Phage Attachment Sites and Controlled Expression of Chromosomal Genes in *Escherichia coli*

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ABSTRACT: Genetic manipulation of cells for desired traits is the most appreciable strategy implemented in the field of bioengineering. However, this approach closely relies on the use of plasmids and is commonly afflicted by the potential problem of plasmid instability and safety caution. Meanwhile, it may also lead to the spread of antibiotic-resistant markers with replicons of plasmids to the environment. However, this issue has long been neglected. In this study, we have addressed these subjects by developing replicon-free and markerless methods for chromosomal insertion of genes and controlled expression of genomic genes in *Escherichia coli*. For the former application, the integration vectors of conditional replication were incorporated with the prophage attachment site and duplicated FRT sites. Their utility was illustrated by site-specific insertion of target genes, the endogenous *dxs* gene and three heterologous genes consisting of *gps*, *crtI*, and *crtB*, fused to T7 promoter into *E. coli* genome. For the latter application, the template vectors for promoter replacement were constructed to carry a DNA cassette containing the T7 promoter linked to a selective marker flanked with the FRT site. Subsequently, it was illustrated by replacement of the native promoter of chromosomal *pckA* by the T7 promoter. Finally, with the aid of FLP recombinase supplied from a helper plasmid, the regions containing replicon and/or selective markers in inserted DNAs were eliminated from integrants for both approaches. As a consequence, the expression of these five genes was subject to control by one response regulator, T7 RNA polymerase, in a regulon way, resulting in a high and stable production of lycopene in the cell. This result indicates the promise of developed methods for genome engineering in *E. coli*.

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Introduction

Escherichia coli stain has been widely accepted as a robust cell employed for industrial production of recombinant proteins, pharmaceuticals, fine chemicals, and bulk commodities. To improve these production processes, it is imperative to engineer producer cells via genetic manipulations to achieve desired traits. In general, producer cells with a new or improved feature can be generated by (i) deletion of undesirable genes, (ii) enhanced expression of key endogenous genes, and (iii) expression of heterologous genes to augment the existing pathways. Plasmids appear to be an indispensable tool for fulfilling these goals. Particularly, they facilitate the establishment of multiple copies of target genes in host cells when the selective pressure is applied. However, for the purpose of pathway engineering, manifold copies of plasmid-encoded genes is usually unnecessary (Jones et al., 2000), and the redundant copy of DNAs can sometimes induce physiological stresses in host cells, consequently leading to segregational loss or internal rearrangements of plasmids (Peredelchuk and Bennett, 1997). In addition, plasmids carrying the antibiotic-resistant determinant are commonly implemented to ensure their maintenance in *E. coli*. It has been well recognized that the use of antibiotics is forbidden for some applications, and the potential risk also exits for the spread of the antibiotic-resistant trait to other microbes in nature (Julian et al., 2001).

Apparently, all these problems arose by using plasmids to achieve desired traits of *E. coli* can be circumvented by

chromosome engineering of the strain based on the recombinase system. With the exploration of λ Red-based recombination system, the approach by disruption of undesirable chromosome genes in *E. coli* has proven very effective and been used extensively (Datsenko and Wanner, 2000). Similarly, the system can be applied to enhance the expression of chromosomal genes, and it begins with the production of a polymerase chain reaction (PCR) DNA. The resulting DNA contains a core region bracketed with two extensions homologous to the regulatory region upstream of the target gene. This core fragment essentially comprises an artificial promoter (sometimes associated with other regulatory elements) linked to an antibiotic-resistant marker. By the act of λ-Red recombinases, the regulatory region of the targeted gene is replaced with the foreign promoter after the occurrence of a crossover event between the homologous regions. As a result, the expression of the chromosomal gene is subject to modulation in a constitutive way (Meynial-Salles et al., 2005; Yuan et al., 2006).

For incorporation of heterologous genes into *E. coli* chromosome, the flippase-based method has been proposed (Martinez-Morales et al., 1999b). This approach relies on the construction of two flanking FRT sites in the suicidal plasmid which carries the guide DNA fused with genes to be inserted. After integration, the antibiotic-resistant gene and plasmid-replication region surrounded by two FRT sites are eliminated by the act of the FLP recombinase provided from a helper plasmid. However, this approach mainly relies on the function of *recA* for directing in vivo recombination which is commonly deemed very inefficient, and it is used for insertion of DNAs with heterologous nature. Moreover, the integrated DNA is sandwiched by two repeated regions of the guide DNA as a result of the homologous recombination, which may result in occasional removal of the inserted DNA by looping out mediated by *recA*.

Recently, Haldimann and Wanner (2001) have developed a series of vectors called CRIM plasmids. The primary feature of CRIM plasmids consists of a conditional-replication origin and a phage attachment (*attP*) site. With the use of a helper plasmid expressing the phage integrase (Int), these plasmids can be integrated into their respective bacterial attachment (*attB*) site in single copy. As illustrated, the integration efficiency of CRIM plasmids is extremely high and integrants are highly stable as well. In addition, this approach is particularly attractive because it allows the site-specific insertion of DNAs into *E. coli* genome. However, the complication still remains when implementing this approach because the replication origin and the antibiotic-resistant marker are retained in the integrated DNA. To eliminate the complication caused by using CRIM plasmids, in this work we have modified these plasmids by bracketing the multiple cloning site (MCS) and an *attP* locus (for phage λ, HK022, ϕ80, P21, and P22) with the FRT site. These integration vectors facilitated the site-specific insertion of genes into *E. coli* chromosome and allowed later elimination of replicons and selective markers. Meanwhile, to achieve the inducible expression of chromosomal genes, a series of template vectors for promoter replacement was also developed. These R6K replicon-based template plasmids essentially contained the T7 promoter in conjunction with an antibiotic-resistant marker flanked by the FRT site. Consequently, the utility of these vectors was illustrated with a great promise by chromosome engineering of *E. coli* to achieve a high and stable production of lycopene. To our knowledge, this is the first case reporting the generation of a lycopene-hyperproducing *E. coli* strain entirely free of replicons and selective markers.

Materials and Methods

Bacterial Strains and Culturing Condition

Manipulation of DNAs was routinely conducted in *E. coli* strain DH5α(pir) whereas strain BL21 (DE3) was engineered for the production of lycopene. Lycopene-producing strains were grown in shake flasks containing Luria-Bertani (LB) medium (Miller, 1972) supplemented with 0.4% glucose while their growth was monitored turbidimetrically at 600 nm (OD_{600}). Recombinant cells were grown for overnight and seeded to have an initial cell density of 0.1 at OD_{600}. The culture was maintained in an orbital shaker set at 200 rpm and 37°C. Upon reaching 0.4 at OD_{600}, the cells were induced for lycopene production by adding 20 μM IPTG.

Plasmid Construction

The plasmids and primers used in this study were listed in Table I. As included in Figure 1A, integration vectors were constructed in several steps. With PCR, the *cat* gene (encoding chloramphenicol acetyltransferase) in pKD3 (Datsenko and Wanner, 2000) was removed with primers RC0371-0372. Meanwhile, the DNA containing MCS and the *attP* site for phage HK022 ($attP_{HK}$) was amplified from pAH68 (Haldimann and Wanner, 2001) using oligomers RC0364-0365. These two PCR DNAs were then digested with *Pst*I–*Stu*I and subsequently ligated together to produce pHK-Ap. To generate pHK-Km and pHK-Cm, the gene encoding β-lactamase (*bla*) in pHK-Ap was eliminated by *Not*I–*Sph*I cleavage and replaced with the *kan* gene (encoding aminoglycoside 3′-phosphotransferase) recovered from pAH120 (Haldimann and Wanner, 2001) and the *cat* gene from pCAH63 (Haldimann and Wanner, 2001), respectively. Moreover, DNAs consisting of MCS and various *attP* sites (for phage ϕ80 ($attP_{Phi}$), P21 ($attP_{P21}$), and P22 ($attP_{P22}$)) were synthesized by PCR using primers RC0364-0365 by complementing pAH153, pAH81, and pAH154 (Haldimann and Wanner, 2001), respectively. After removal of the corresponding DNA fragment from pHK-Km and pHK-Cm by *Pst*I–*Stu*I digestion, the remaining vector scaffolds were spliced with these PCR-generated DNAs to give pPhi-Km, pPhi-Cm, pP21-Km, pP21-Cm, pP22-Km, and pP22-Cm. Finally, pLamda-Km and

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

	Relevant characteristics	Sources
Strain		
DH5α(pir)	*deoR endA1 gyrA96 hsdR17 supE44 thi1 recA1 lacZM15 λpir*	Lab collection
BL21(DE3)	F^- *dcm gal ompT hsdS*(r_B^- m_B^-) (DE3)	Novagen
BL21-Crt	BL21(DE3) *gps*$^+$ *crtI*$^+$ *crtB*$^+$	This study
BL21-CrtD1	BL21-Crt with an additional chromosomal copy of *dxs* fused to the T7 promoter	This study
BL21-CrtD2	BL21-Crt with native *dxs* fused to the T7 promoter	This study
BL21-CrtD1K	BL21-CrtD1 with native *pckA* fused to the T7 promoter	This study
Plasmid		
pHK-Km	Integration vector with $attP_{HK}$ and *kan*$^+$	This study
pHK-Cm	Integration vector with $attP_{HK}$ and *cat*$^+$	This study
pPhi80-Km	Integration vector with $attP_{Phi}$ and *kan*$^+$	This study
pPhi80-Cm	Integration vector with $attP_{Phi}$ and *cat*$^+$	This study
pP21-Km	Integration vector with $attP_{P21}$ and *kan*$^+$	This study
pP21-Cm	Integration vector with $attP_{P21}$ and *cat*$^+$	This study
pP22-Km	Integration vector with $attP_{P22}$ and *kan*$^+$	This study
pP22-Cm	Integration vector with $attP_{P22}$ and *cat*$^+$	This study
pLamda-Km	Integration vector with $attP_{\lambda}$ and *kan*$^+$	This study
pLamda-Cm	Integration vector with $attP_{\lambda}$ and *cat*$^+$	This study
pCP20	Helper plasmid expressing FLP with *bla*$^+$	Datsenko and Wanner (2000)
pKD46	Helper plasmid expressing λ-Rad with *bla*$^+$	Datsenko and Wanner (2000)
pKD-T7	Template vector containing the *kan*-P_{T7} cassette	This study
pKD-T7rbs	Template vector containing the *kan*-$P_{T7/RBS}$ cassette	This study
pTD-T7	Template vector containing the *tetA*-P_{T7} cassette	This study
pTD-T7rbs	Template vector containing the *tetA*-$P_{T7/RBS}$ cassette	This study
pP21-Crt	pP21-Cm with *gps crtI crtB*	This study
pHK-dxs	pHK-Cm with *dxs*	This study
pINT-ts	Helper plasmid expressing phage λ Int with *bla*$^+$	Haldimann and Wanner (2001)
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)
pA123	Helper plasmid expressing phage ϕ80 Int with *bla*$^+$	Haldimann and Wanner (2001)
pAH130	Helper plasmid expressing phage P22 Int with *bla*$^+$	Haldimann and Wanner (2001)
Primer[a]		
RC0371	GCGCCTGCAGAATGAAGTTCCTATTCCGAAG (*Pst*I)	
RC0372	CCTTCTGCGAAGTGATCTTCCGTC	
RC0364	CTGACATGGGAATTAGCCATGG (*Nco*I)	
RC0365	CTTGCATGCCTGCAGGTCG (*Pst*I)	
RC0419	ATAGGGAGACCCTAGCTTTGCACTGGATTG	
RC0420	AGTGAGTCGTATTTAATTCCGGGGATCCGTC	
RC0431	AACGCTGCAGGAGATCTCGATCTCGATCC (*Pst*I)	
RC0432	ATCCGGATCCAGTTCCTCCTTTCAG (*Bam*HI)	
RC0444	TGTTTAACTTTAAGAAGGAG*CTAGCTTTGCACTGGATTGC*	
RC0445	AAATTATTTCTAGAGGGAAACCGTTGT*GGTCTCCCTATAGTGAGTCG*	
RC0460	TAAGGGTACCTTGAAGTTCCTATTCCGAAG	
RC0461	GAAAGGCATGCTAACAGCACAAG	
RC0471	AAGGTTGAACATATGGATCGATTTCAGAGG (*Nde*I)	
RC0472	ATTTAAGCTTCCGCTCTTCACTTTTTTCTTGTAAC (*Hind*III)	
RC0473	TTCCAAGCTTAGGAGATAAAGGATGAAAAAAACCGTTG (*Hind*III)	
RC0474	AACCCTCGAGCTAAACGGGACGCTGCCAAAG (*Xho*I)	
RC0496	ATCCGGATCCAGTTCCTCCTTTCAG	
RC04142	GCCCATATGAGTTTTGATATTGCC (*Nde*I)	
RC04143	GTGGCCATGGGATTATGCCAGCCAG (*Nco*I)	
RC0501	GGACTACATCATCCAGCGTAATAAATAAACAATAAGTATTAATAGGCCC*GTGTAGGCTGGAGCTGCTTC*	
RC0502	GTCGACCAGTGCCAGGGTCGGGTATTTGGCAATATCAAAACTCATCAG *AGTGCAAAGCTAGCTCCTTC*	
RC0503	TATTTCTGCGATAATAGCAACCGTTTCGTGACAGGAATCACGGAGTTTTT*GTGTAGGCTGGAGCTGCTTC*	
RC0504	CTTAGCCAATATGTATTGCCTGAATAGTAAAGTCTTTTTGGGGGTGTTAAC*AATCCAGTGCAAAGCTAGG*	
T1	as RC0372	
T2	as RC0371	
T3/gps	as RC0471	
T4/crtB	as RC0474	
T4/dxs	as RC04143	
Pm1	GTGTAGGCTGGAGCTGCTTC	
Pm2	AGTGCAAAGCTAGCTCCTTC	
Pm3	AATCCAGTGCAAAGCTAGG	
H3/dxs	GATTATGCCAGCCAGGCCTG	
H3/pck	GGGTGTCGGTGTATTTATCG	

[a]Restriction sites included within the primers are underlined and indicated on the right in parentheses. The Pm1, Pm2, or Pm3 sequence appearing in primers RC0501, RC0502, RC0503, and RC0504 is italicized.

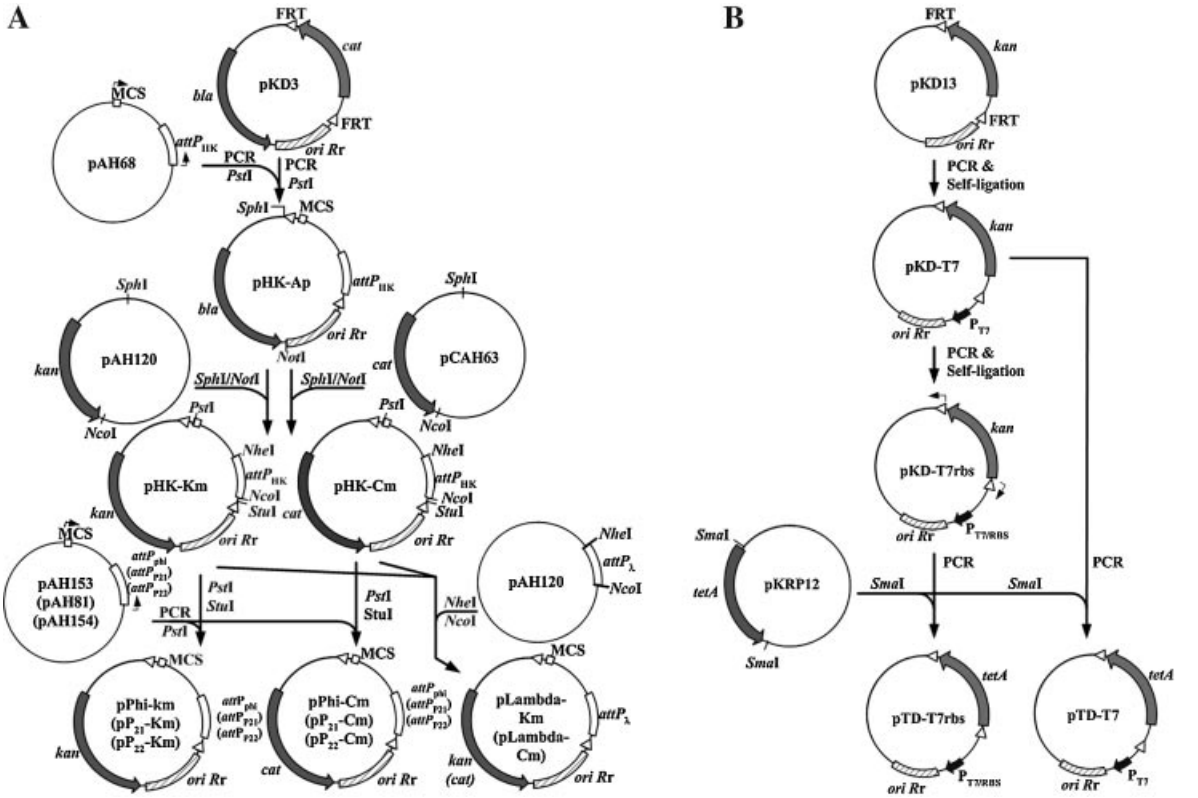


Figure 1. Schematic illustration of the procedure for the construction of integration plasmids (**A**) and template vectors (**B**). As a result of plasmid construction, unique sites remained within MCS of integration plasmids including *Pst*I, *Sal*I, *Xba*I, *Bam*HI, *Sma*I, *Kpn*I, *Sac*I, and *Eco*RI. *oriR*r, conditional-replication R6K origin; P_{T7}, T7 promoter; $P_{T7/RBS}$, T7 promoter with RBS.

pLamda-Cm were produced by replacement of $attP_{HK}$ on pHK-Km and pHK-Cm with the *attP* site for phage λ ($attP_{\lambda}$) recovered from pAH120 (Haldimann and Wanner, 2001) treated with *Nco*I–*Nhe*I.

The template vectors for promoter replacement were constructed as follows (Fig. 1B). To reconstitute the T7 promoter, primers RC0419-0420 with 5′-extensions were synthesized to comprise the sequence complementary to pKD13. On incorporation of the primers, pKD13 was modified with the generation of staggered nicks by *Pfu* DNA polymerase. Followed by treatment with *Dpn*I, the PCR DNA was self-ligated and used for transformation into *E. coli* strain DH5α. One resulting plasmid was recovered and sequenced for the confirmation of the T7 promoter, designated pKD-T7. In a similar fashion, pKD-T7rbs was produced using oligomers RC0444-0445 with complementation to pKD-T7. The added ribosome binding site (RBS) on pKD-T7rbs was verified by DNA sequencing. Consequently, the T7 promoter and the T7 promoter with RBS were created on template vectors with the sequence of AAT*TAATACGACTCACTATA*GGGGAGACC and AAT*TAATACGACTCACTATA*GG GGAGACCACAACGG-TTTCCCTCTAGAATATTAACTTTAAGAAGGAG. The sequence of the T7 promoter and RBS was italicized and underlined, respectively. Moreover, the *kan* marker on pKD-T7 and pKD-T7rbs was removed by PCR using primers RC0460-0461. The reaming plasmid DNAs were spliced with a DNA fragment containing tetracycline-resistance gene (*tetA*), recovered from pKRP12 (Reece and Phillips, 1995) by *Sma*I digestion, to obtain pTD-T7 and pTD-T7rbs. Finally, these template vectors mainly contain the DNA cassette consisting of a FRT-flanked antibiotic marker linked to the T7 promoter with (Ab-$P_{T7/RBS}$) or without RBS (Ab-P_{T7}). The DNA sequences of plasmids constructed in this work will be provided upon request.

Chromosomal Integration of Genes and Removal of Replicon With the Marker

The *gps* gene (encoding geranyl-geranly diphosphate (GGPP) synthase) of *Archaeoglobus fulgidus* was synthesized from plasmid pCW3 (Wang et al., 1999) by PCR using primers RC0471-0472. Followed by *Nde*I–*Hin*dIII digestion, the PCR DNA was incorporated into the corresponding sites of pET-20bI (Wang et al., 2004) to produce pET-gps. Meanwhile, two cistrons comprising *crtI* and *crtB* were amplified from *Erwinia herbicola* Eho10 (Perry et al., 1986)

with primer RC0473-0474, and the resulting DNA was then ligated into the *Hin*dIII–*Xho*I site of pET-gps to give pET-Crt. With primers RC0431-0496, the gene cluster flanked by the T7 promoter and the T7 transcription terminator was produced by PCR and cloned into the *Pst*I–*Sma*I site of pP21-Cm to generate pP21-Crt. For cloning of the *dxs* gene, PCR was carried out with primers RC04142-04143 using strain BL21 (DE3) chromosome as template. The PCR DNA was pretreated with *Nde*I–*Nco*I digestion and then incorporated into pET-20bI to produce pET-dxs in which the *dxs* gene was placed under the control of the T7 promoter. Followed by another PCR, the DNA containing *dxs* gene bracketed by the T7 promoter and terminator was generated with primers RC0431-0432 from pET-dxs. Finally, the resulting DNA was subcloned into the *Pst*I–*Bam*HI site of pHK-Cm to give pHK-dxs.

Strain BL21 (DE3) and its derivatives were made competent for DNA transformation using calcium (Miller, 1972). Strains carrying a helper plasmid 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_{600}. The cells were then made competent and transformed with integration vectors containing genes to be inserted (pP21-Crt or pHK-dxs). The resulting cells spread onto selective LB agar plates were tested for loss of the helper plasmid (conferring ampicillin sensitivity) and stable integration (conferring either kanamycin or chloramphenicol 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.

Promoter Replacement of Chromosomal Genes and Elimination of Markers

Strains carrying pKD46 (Datsenko and Wanner, 2000) expressing the λ-Red recombinase were grown in LB medium with 1 mM L-arabinose but without antibiotics at 30°C. After reaching OD_{600} of 0.6, the cells were concentrated by 100-fold and washed two times with ice-cold 10% glycerol. The PCR DNAs (100 ng) amplified from the template vectors were gel-purified and electroporated into competent cells, and the electro-shocked cells were then incubated in SOC medium at 37°C for 2 h. The resulting cells were spread onto selective LB medium with the selection for kanamycin or tetracycline resistance at 39°C and subsequently examined for ampicillin sensitivity (loss of pKD46). Finally, integrants were transformed with the FLP-expressing helper plasmid pCP20 and processed in a similar way for the removal of selective markers as described above.

Analytical Methods

The lycopene content of cells was determined following the previous method (Kim and Keasling, 2001). In brief, cell cultures were periodically harvested by centrifugation and rinsed with saline water. Followed by immersing in acetone at 55°C in the dark, cells were disrupted and centrifuged for the recovery of the supernatant. The supernatant solution was then measured at 470 nm with a spectrophotometer, and the absorbance obtained for the sample was compared to a lycopene standard (Sigma, St. Louis, MO).

Based on the reaction mediated by 1-deoxy-D-xylulose-5-phosphate (DXP) synthase (Dxs), the Dxs activity was determined by measuring the disappearance of pyruvate with high-performance liquid chromatography (HPLC). The enzyme reaction mixture consisted of 40 mM Tris–HCl (pH 7.5), 2.5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 1 mM thiamin diphosphate, 10 mM sodium pyruvate, and 20 mM glyceraldehyde 3-phosphate in a final volume of 200 μL. The reaction was started by adding 0.25 mg of the cell-free extract (CFX) into the reaction solution at 37°C for 15 min and quenched by heating at 80°C for 5 min. Prior to HPLC analysis, the reaction solution was passed through a 0.2-μm filter and applied to the ion-exchange column (Transgenomic) with a mobile phase containing 0.0085 N sulfuric acid pumped at 0.4 mL/min. The eluate was then monitored at 280 nm at 40°C. The CFX was prepared by harvesting cells with centrifugation and resuspended in the Tris–HCl buffer. After disruption by sonication, cell debris was discarded upon centrifugation while the supernatant was recovered for the determination of its concentration using Bio-Rad dye reagent with bovine serum albumin as the protein standard. In addition, the phosphoenolpyruvate (PEP) carboxykinase (Pck) activity was assayed exactly following our previous reports (Chao et al., 2003a,b). The specific enzyme activity (U/mg) for both Dxs and Pck was defined as μmol of pyruvate and oxaloacetate consumed per min (U) per mg protein, respectively.

Results

Chromosomal Insertion of Heterologous Genes

The general procedure for using integration vectors (Fig. 1A) was illustrated in Figure 2. This approach allows chromosomal insertion of genes and later elimination of most of the integrated plasmid DNA consisting of the selective marker and R6K-replication origin. It starts with directly subcloning the promoter-bearing genes into the MCS of integration vectors. Subsequently, recombinant plasmids are transformed into a *pir* (encoding the replication protein for the R6K origin)-free strain carrying a helper plasmid expressing a specific phage Int. Upon temperature upshift, the phage Int under control of the heat-inducible λP_R promoter is forced to produce, while the helper plasmid can be simultaneously cured from the host strain due to the thermo-sensitive nature of the replication

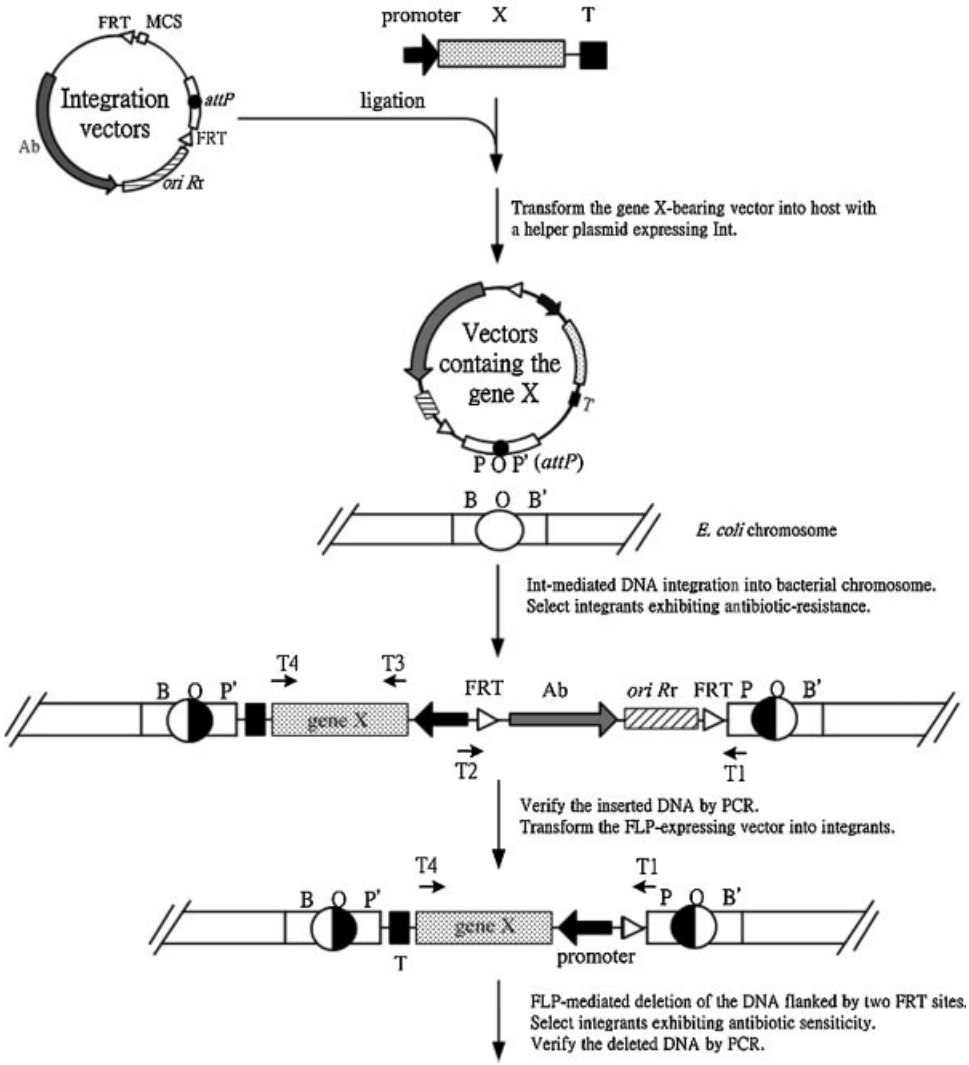
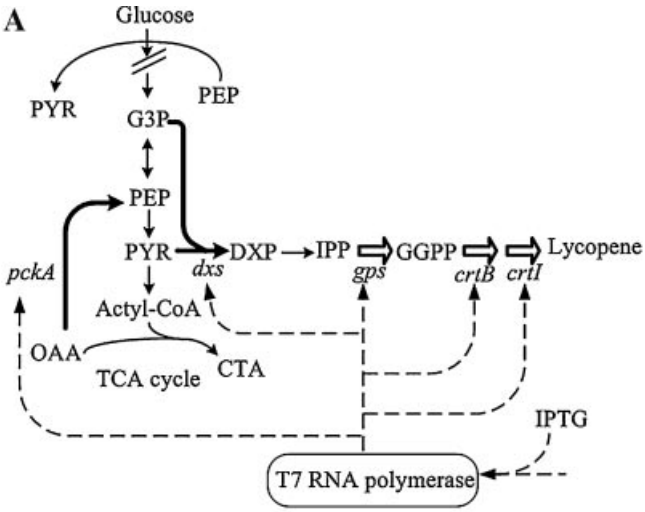


Figure 2. Schematic illustration of the utility of integration vectors for insertion of genes into *E. coli* chromosome. For chromosomal insertion, a gene of interest (X) with a promoter and a transcription termination site (denoted as T) was first incorporated into the MCS of integration plasmids. Subsequently, the resulting plasmid was transformed into *E. coli* strain BL21 (DE3) bearing a helper plasmid expressing a specific Int. Helper plasmids producing respective phage Int were listed in Table I. Refer to text for more details.

protein (Haldimann and Wanner, 2001). Aided by the phage Int, the *attP* (denoted by POP′) and *attB* (denoted by BOB′) sites are crossed over. Integrants with the insertion of DNA is then screened for exhibiting resistance to the antibiotic. Further verification of the integrated DNA is carried out with in situ PCR using primers T1–T2 (Table I). For heterologous genes of insertion, additional PCR is performed using primers T3–T4. To remove the plasmid DNA flanked by the FRT site, plasmid pCP20 is transformed into the integrants. Like the phage Int-producing helper plasmid, pCP20 expressing FLP is conditionally unstable at high temperatures (Datsenko and Wanner, 2000). After exposure to heat, the cell is examined for sensitivity to the antibiotic, and the DNA deletion event is further confirmed by in situ PCR with primers T1–T4.

As indicated in Figure 3A, the metabolic pathway of *E. coli* can be extended to lycopene production with the recruitment of three heterologous genes consisting of *gps*, *crtI*, and *crtB*. To illustrate the utility of integration vectors, plasmid pP21-Crt containing the *crt* gene cluster (*gps-crtI-crtB*) under the control of the T7 promoter was constructed as described. According to the procedure depicted in Figure 2, pP21-Crt was transformed into *E. coli* strain BL21(DE3) with pAH121 expressing phage P21 Int (Haldimann and Wanner, 2001). After selection for integrants exhibiting chloramphenicol resistance but ampicillin sensitivity (indicating the loss of the helper plasmid pAH121), in situ PCR was implemented to verify the inserted DNA in integrants. As shown in Figure 3B, the DNA containing the R6K-replication origin, the selective marker, and the *crt* gene cluster were identified by PCR with expected sizes using the pairs of primers T1–T2, T3/gps–T4/crtB, and T1–T4/crtB (Table I). Later removal of the antibiotic-resistant determinant along with the replication origin was aided by FLP



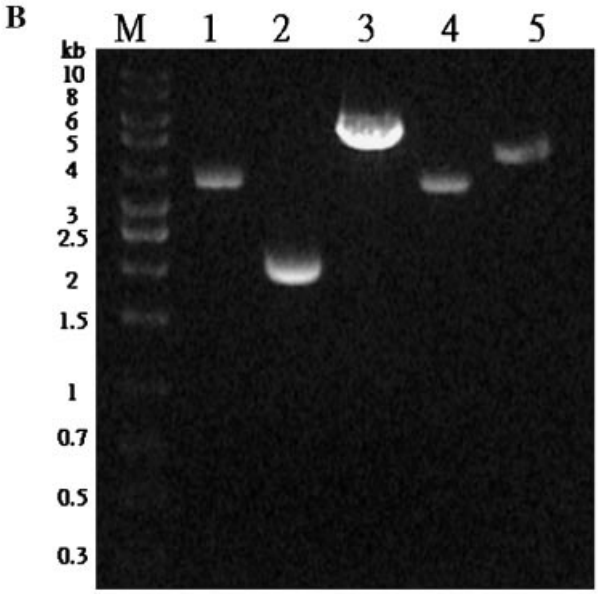


Figure 3. **A**: Metabolic pathway leading to the synthesis of lycopene in *E. coli*. The metabolic pathway in *E. coli* was reconstructed for the production of lycopene. Like a regulon, five genes, *gps*, *crtB*, *crtI*, *dxs*, and *pckA*, were engineered to be regulated by one response regulator, the T7 RNA polymerase, in response to IPTG (as indicated by broken lines). G3P, glyceraldehyde 3-phosphate; PEP, phosphoenolpyruvate; PYR, pyruvate; OAA, oxaloacetate; CTA, citric acid; DXP, 1-deoxy-D-xylulose-5-phosphate; IPP, isopentenyl diphosphate; GGPP, geranylgeranyl diphosphate. **B**: Analysis of the event of gene insertion and DNA removal by agarose gel electrophoresis. Refer to Figure 2, an integrant carrying the inserted *crt* gene cluster was verified by PCR with primers T1–T2 and T3/gps-T4/crtB (Table I). The latter primer set has the sequence complementary to the 5′-teminus of *gps* and the 3′-terminus of *crtB*. Furthermore, the event with the elimination of the replication origin and the marker was confirmed with the primer T1–T4/crtB. Keys: M, the DNA marker; lane 1, confirmation of the inserted *crt* gene cluster (3.5 kb) by primers T3/gps-T4/crtB; lane 2, confirmation of the inserted DNA containing the replication origin and the marker (2 kb) by primers T1–T2; lane 3, confirmation of the inserted DNA containing the replication origin, the marker, and the *crt* gene cluster (5.5 kb) by primers T1–T4/crtB; lane 4, confirmation of the inserted *crt* gene cluster (3.5 kb) by primers T3/gps-T4/crtB after the DNA deletion; lane 5, the remained plasmid DNA residue with the inserted *crt* gene cluster (4.6 kb) by primers T1–T4/crtB after the DNA deletion.

expressed from pCP20. Subsequently, integrants susceptible to both chloramphenicol (loss of marker) and ampicillin (loss of pCP20) were examined for the elimination of the marker and the replication origin. Using PCR with primers T1–T4/gps and T3/gps–T4/crtB (Table I), it revealed the presence of the *crt* gene cluster, without the replicon and the marker, in one resulting strain (designated BL21-Crt). This strain carrying a chromosomal copy of the *crt* gene cluster controlled by the T7 promoter was employed for further study.

Further investigation of strain BL21-Crt for lycopene production was carried out in shake flasks. The cultures were induced by adding various IPTG (10, 20, 30, 50, 70, and 100 μM) and lycopene production was determined. After fermentation for 36 h, the maximal production of lycopene was obtained for the cell induced with 20 μM IPTG. In comparison with the uninduced cell, the induced BL21-Crt strain displayed an orange color as an indicative of lycopene production.

Chromosomal Insertion of Native Genes

As disclosed from the lycopene-synthetic pathway (Fig. 3A), the first committed step for the formation of DXP from two precursors metabolites, pyruvate and glyceraldehyde 3-phosphate, is catalyzed by Dxs (encoded by *dxs*). It is evident that an elevated level of DXP would be advantage for the production of lycopene. Therefore, in the hope of enhancing the Dxs activity, integration plasmid pHK-dxs was constructed to carry *dxs* fused to the T7 promoter (P_{T7}::*dxs*) as described. Based on the procedure depicted in Figure 2, genomic integration of the DNA containing P_{T7}::*dxs* was conducted by transformation of pHK-dxs into the lycopene-producing strain BL21-Crt with the helper plasmid pAH69 expressing phage HK022 Int (Haldimann and Wanner, 2001). The DNA insertion event was confirmed by PCR using primers T1–T2 and T1–T4/dxs (data not shown), where primer T4/dxs contains a sequence complementary to the 3′-terminus of *dxs* (Table I). Subsequent removal of the replicon and the antibiotic marker was carried out by FLP expressed from pCP20. After selection for the cell exhibiting chloramphenicol and ampicillin sensitivity, one resulting strain (designated BL21-CrtD1) was obtained and verified by PCR using primers T1–T4/dxs (data not shown). Furthermore, strain BL21-CrtD1 was investigated for lycopene production in a similar way. Upon IPTG induction, the cell produced 10 times more lycopene than strain BL21-Crt did after fermentation for 36 h. As shown in Table II, this increase in lycopene production was correlated with the rise of Dxs activity. The result indicates the success of this approach in introducing an extra copy of *dxs* into *E. coli* genome, and its expression is responsive to IPTG.

Controlled Expression of Chromosomal Genes

To achieve the controlled expression of chromosomal genes, the general protocol for the use of the template vectors

Table II. Lycopene production, biomass yield, and enzyme activity for recombinant strains.

Strain	Dxs activity (U/mg)	Pck activity (U/mg)	Lycopene (mg/L)	Biomass (g DCW/L)
BL21-Crt	0.4 (1)	0.1 (1)	5 (1)	1.8
BL21-CrtD1	3.2 (8)	ND	55 (11)	2.3
BL21-CrtD2	1.0 (2.5)	ND	10 (2)	1.9
BL21-CrtD1K	3.2 (8)	0.6 (6)	100 (20)	2.6

Shake-flask cultures of recombinant strains were carried out and induced for lycopene production. Cells were harvested for determination of the enzyme activity and lycopene production after fermentation for 24 and 36 h, respectively. The enzyme activity and lycopene yield for recombinant *E. coli* strains were normalized and given in parentheses.

DCW, dry cell weight; ND, not determined.

(Fig. 1B) was outlined in Figure 4. First, synthetic oligomers are designed to contain the priming sequences (Pm1, Pm2, and Pm3) of 20 bases complementary to template vectors and the extension sequences (H1 and H2) of 40–60 bases homologous to the targeted locus on chromosome. As a result, the DNA product consisting of the DNA cassette (Ab-P_{T7} or Ab-$P_{T7/RBS}$) bracketed with two homology extensions is produced by PCR using the synthetic primers. After purification, the PCR DNAs are transformed into a host strain with the helper plasmid pKD46 expressing the λ-Red recombinases. Upon elimination of pKD46 by heat, integrants exhibiting resistance to the antibiotic are examined for the inserted DNA cassette Ab-P_{T7} or Ab-$P_{T7/RBS}$ using in situ PCR with primers Pm1–Pm2 and Pm1–Pm3, respectively. To remove the selective marker, the FLP-expressing plasmid pCP20 is introduced into the cell and resulting transformants are screened for showing sensitivity to antibiotic (indicating the marker removal and the loss of pCP20) after exposure to heat. Finally, the event with the removal of markers is verified by PCR using primers Pm1-H3.

The utility of template vectors for promoter replacement was first demonstrated to achieve the controlled expression of the chromosomal *dxs* by the T7 promoter. As revealed from the *E. coli* genome sequence, the gene locates in an operon consisting of four cistrons, *xseB*, *ispA*, *dxs*, and *xajO*. For independent regulation, two synthetic primers RC0501-0502 (Table I) were designed to fuse the DNA cassette Ab-$P_{T7/RBS}$ with the structural gene of *dxs*. In essence, the former oligomer contains the Pm1 sequence and the H1 extension sequence corresponding to the 3′-terminus of *ispA* while the latter comprises the Pm3 sequence and the H2 extension sequence homologous to the 5′-terminus of *dxs*. Using pKD-T7rbs as the template, primers RC0501-0502 were used to synthesize the tripartite gene fusion consisting of the *kan*-$P_{T7/RBS}$ cassette sandwiched by the truncated parts of *ispA* and *dxs*. After transformation of the linear DNA into strain BL21-Crt bearing pKD46, integrants showing resistance to kanamycin were selected and confirmed by PCR using primers Pm1–Pm3 (data not shown). Later removal of the marker was aided by FLP and verified by PCR with primers Pm1-H3/dxs (data not shown). The resulting strain was designated BL21-CrtD2 and subsequently employed for the production of lycopene in shake flasks. As a result, lycopene production was doubled for strain BL21-CrtD2 relative to that by strain BL21-Crt (Table II). The enhanced production yield was reflected by a 2.5-fold increase in the Dxs activity for strain BL21-CrtD2. Together with the result obtained for strain BL21-CrtD1 (Table II), the synthesis of lycopene appears to be controlled by the Dxs in the pathway.

It is expected that the determining step for lycopene production is probably moved upwards after the elimination of bottlenecks in the downstream pathway. As revealed in Figure 3A, the reaction catalyzed by Pck (encoded by *pckA* gene) could recycle the carbon flux from OAA in the TCA cycle to PEP, which in turn provides the two precursor metabolites (PYR and G3P) for the synthesis of lycopene (Fig. 3A). Therefore, an attempt for up-regulated expression of chromosomal *pckA* gene was made by replacement of its native promoter with the T7 promoter. Likewise, primers RC0503-0504 (Table I) were synthesized to contain homologous sequences complementary to the upstream of the regulatory region and 5′-terminus of the *pckA* gene. As a consequence of PCR, the DNA composed of the *kan*-P_{T7} cassette fused with the two homology extensions was produced from the template vector pKD-T7 and transformed into strain BL21-CrtD1 by electroporation. Following the procedure depicted in Figure 4, an integrant bearing the inserted DNA cassette Ab-P_{T7} was verified by PCR with primers Pm1–Pm2 (data not shown). Followed by the elimination of the antibiotic marker, one resulting strain (designated BL21-CrtD1K) was obtained and the deleted DNA was further confirmed by PCR using primers Pm1-H3/pck (data not shown). In a similar manner, the shake-flask culture of strain BL21-CrtD1K was carried out and examined for lycopene production. As a result, it showed that the lycopene level could be enhanced by 20 folds for strain BL21-CrtD1K (overexpressing *dxs* and *pckA*) without compromising its growth as compared to that for strain BL21-Crt (Fig. 5). Moreover, strain BL21-CrtD1K exhibited sixfold higher Pck activity over the wide-type level. Taken together, it indicates the success of the approach in controlling the expression of chromosomal *pckA* by the recruited T7 promoter.

Discussion

The approach for integration of heterologous genes into *E. coli* chromosome has been mainly performed by means of

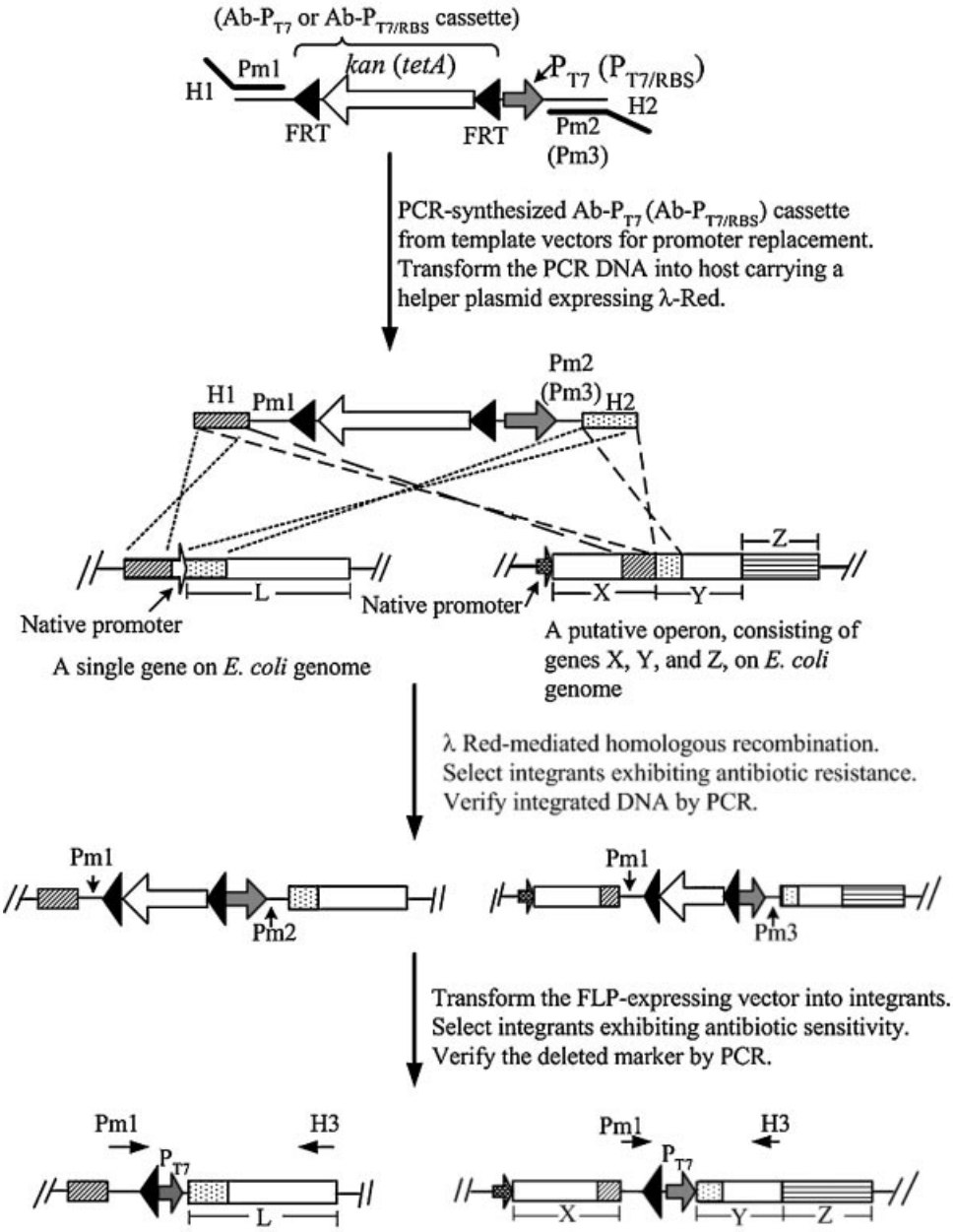


Figure 4. Schematic illustration of the utility of the template vectors for replacement of chromosomal gene promoters with the T7 promoter. The controlled expression of the chromosomal gene could be achieved by replacement of the native promoter of a gene with the T7 promoter or insertion of the T7 promoter into the gene within an operon. Primers Pm1–Pm2 and Pm1–Pm3 contain the sequence complementary with template vectors pKD-T7/pTD-T7 and pKD-T7rbs/pTD-T7rbs (Table I), respectively. For verification by PCR, primer H3 corresponding to 3′-terminus of the targeted gene was used in conjunction with primer Pm1. Refer to text for more details.

modified transposons (de Lorenzo and Timmis, 1994) and conditional-replication plasmids (Le Borgne et al., 1998; Link et al., 1997). For the former method, the chromosomal locus integrated by alien genes appears to be random and multiple copies of inserted genes sometimes appear. The latter approach lies in the use of an endogenous DNA as a guide to direct the insertion of foreign genes into destined loci at the nonpermissive condition. In spite of their feasibility, these methods still raise criticisms for the creation of integrated DNAs in the form of replicons and transposable elements associated with selective markers. This will complicate subsequent constructions when cycling improvements are required and, in particular, possess a potential threat of cross contaminations to the environment. However, these issues have long been neglected. Therefore, in this study we have developed replicon-free and markerless methods for efficient insertion of target genes into phage attachment sites of *E. coli* chromosome. In addition to foreign genes, the approach allows chromosomal integration of endogenous genes, and this feature is particularly helpful

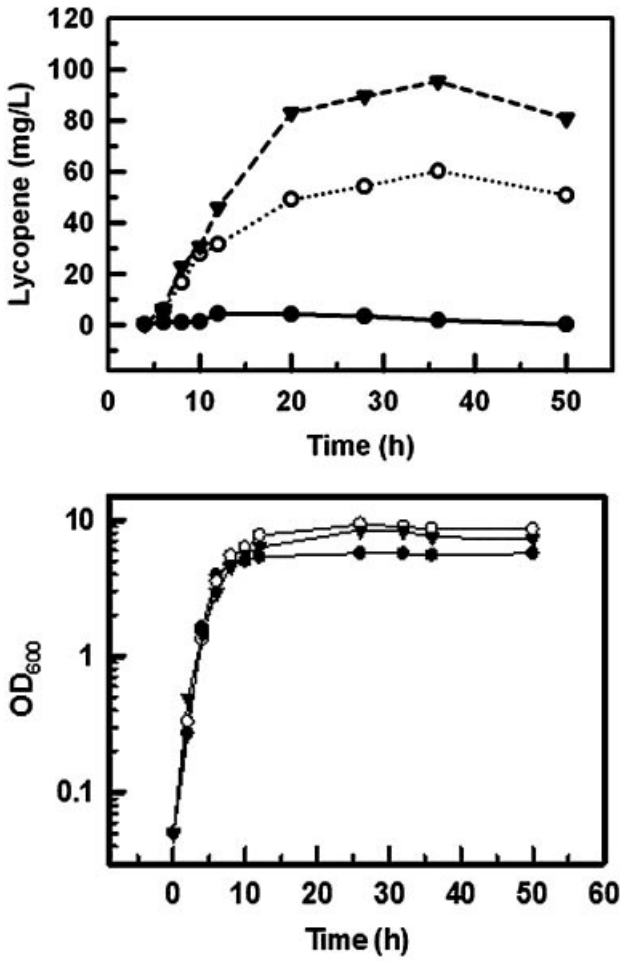


Figure 5. Lycopene production profile (top) and growth curve (bottom) for producer cells. As described, strains BL21-Crt, BL21-CrtD1, B21-CrtD2, and BL21-CrtD1K were grown in shake flasks containing LB medium plus 0.4% glucose. Upon induction by IPTG, cells were sampled for determination of the lycopene yield and their growth was monitored along the time course. The results were taken from three independent experiments with the standard deviation less than 10%. For conciseness, the data obtained for strain BL21-CrtD2 were not shown. (▼) strain BL21-CrtD1K; (○) strain BL21-CrtD1; (●) strain BL21-Crt.

when in situ enforced expression of genomic genes is not properly applicable (see below). As an illustrative example, the site-directed integration of the heterologous *crt* gene cluster and the native *dxs* gene into *E. coli* chromosome were conducted for lycopene production. We found that hundreds of cell forming units (CFUs) on the selective agar plates could be usually obtained simply employing the calcium-based transformation protocol of plasmids. By PCR, more than 90% of cells from tens of CFUs randomly picked were verified as positive integrants irrespective of endogenous and foreign genes for insertion.

To regulate the expression of the key endogenous genes (*dxs* and *pckA*) in the pathway (Fig. 3A), we have also constructed the template plasmids for replacement of native gene promoters with the T7 one. Particularly, in the case of using glucose as the sole substrate, this would permit the expression of *pckA* which is subject to catabolite repression (Goldie, 1984). T7 promoter is chosen for utilization because it is known for its strong strength and specifically requires T7 RNA polymerase for activation (Studier and Moffatt, 1986; Tabor and Richardson, 1985). In addition, the library of promoter variants with a wide range of strength is also available (Imburgio et al., 2000). This appears to be attractive for use once the issue of tuning the expression of genomic genes is addressed (Alper et al., 2005; Meynial-Salles et al., 2005). As previously reported, an optimal expression level of chromosomal genes could be obtained by assessing the responsive effect of their activities from the promoter library on the cell phenotype (Alper et al., 2005). Nevertheless, the challenge still remains that the expression of certain chromosomal genes is not simply and explicitly regulated at the transcriptional level. As revealed here, the T7 promoter with the consensus RBS site was placed in situ to direct the expression of chromosomal *dxs* in strain BL21-CrtD2. It was found that the Dxs activity was slightly increased (Table II). In contrast, by introduction of an additional copy of *dxs* driven by the T7 promoter into the chromosome, strain BL21-CrtD1 exhibited a much higher Dxs activity, accordingly resulting in a burst increase in lycopene production (Fig. 5 and Table II). The discrepancy in *dxs* gene expression level for both cases might reflect the dependence of gene expressions on the insertion site in bacterial genome as previously suggested (Sousa et al., 1997; Wang et al., 2004). In addition, an effect exerted by the intergenic region in the operon on the expression of *dxs* might be likely as well (Kimura et al., 2005). Indeed, it is inevitable to achieve in situ control of the expression of upstream genes in operons by the approach of promoter replacement without affecting that of downstream genes. Once this becomes the subject of concern, the genes in the operon should be individually targeted for regulation like *dxs* as illustrated here by the developed integration method. Therefore, the caution should be taken when using the method for promoter replacement developed in this study or by others (Alper et al., 2005; Meynial-Salles et al., 2005).

An attractive feature of this study is to construct an array of target genes controllable by one response regulator (Fig. 3A). This is based on the characteristic of the T7 promoter with the specific requirement for T7 RNA polymerase. Acting like a regulon, an exclusive expression of the gene array is achieved in response to IPTG which serve as an inducer for directed production of T7 RNA polymerase, therefore reducing the competition for the host RNA polymerase (Tabor and Richardson, 1985). This strategy would allow separation of the product production phase from the cell growth phase, particularly useful for the case where the product is toxic to cells. Consequently with the reconstructed pathway, the engineered strain free of plasmids was induced to largely produce lycopene (100 mg/L), a yield comparable to that by the approach of dynamic metabolic control engineering (Farmer and Liao, 2000), without jeopardizing its growth (Fig. 5). As reported by the study, the convention way by on/off regulation of pathway genes is not

optimized for the balanced adjustment of cellular metabolic flux, thereby leading to very low production of the desired metabolite (lycopene) and growth retardation of cells (Farmer and Liao, 2000). Although the strain and culturing condition are not similar, the result shown by our developed strain deprived of plasmids seems to be in contrast to theirs. Indeed, their lycopene-producing strain was constructed to carry three different plasmids for expressing multiple genes. It is highly likely that their strain was afflicted by the plasmid burden. This in turn may alter host cell physiology to perturb the engineering purpose as reported in literatures (Birnbaum and Bailey, 1991; Martinez-Morales et al., 1999a), and perhaps the way by on/off control of expression would exacerbate such an effect. Furthermore, with the illustration of a single copy of target genes and low dosage of inducer in our strain, the result suggests that a massive amount of key enzymes in the pathway is not required to achieve a high-level production of lycopene.

In conclusion, we have constructed a lycopene hyperproducer *E. coli* strain without carrying the replicon and the selective marker. This ''clean'' producer strain was very stable by examination of its lycopene-producing capacity after 5 sequential transfers. As with the successful illustration, it is conceivable that the proposed methods can be extended to the other metabolite and therapeutic protein production where a cleaner production process is concerned.

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