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Metabolic Engineering 7 (2005) 10–17
METABOLIC ENGINEERING
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Directed evolution of copy number of a broad host range plasmid for metabolic engineering

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Received 3 March 2004; accepted 19 May 2004
Available online 7 January 2005

Abstract

Random mutagenesis and directed evolution has been successfully used to improve desired properties of enzymes for biocatalysis and metabolic engineering. Here we employ the method to increase copy number of a pBBR-based broad host range plasmid, which can be used to express desired enzymes in a variety of microbial hosts. Localized random mutagenesis was performed in the replication control region of a pBBR-derived plasmid containing a β-carotene reporter. Mutant plasmids were isolated that showed increased β-carotene production. Real-time PCR analysis confirmed that the copy number of the mutant plasmids increased 3–7 fold. Sequence of the 10 mutant plasmids indicated that each plasmid contained single or multiple mutations in the *rep* gene or the flanking regions. Single amino acid change of serine to leucine at codon 100 of the replication protein and single nucleotide change of C to T at 46 bp upstream of the *rep* gene caused the increase of plasmid copy number. The utility of the mutant plasmids for metabolic engineering were further demonstrated by increased β-carotene production, when an isoprenoid pathway gene (*dxs*) was co-expressed on a compatible plasmid. The mutant plasmids were tested in *Agrobacterium tumefaciens*. Increase of plasmid copy number and β-carotene production was also observed in the non-*Escherichia coli* host.

Keywords: Broad host range plasmid; Replication protein; Plasmid copy number; Carotenoids; Error-prone PCR

1. Introduction

The tools of random mutagenesis and directed evolution have been widely applied to enzyme engineering to improve properties of enzymes including stability (Zhao and Arnold, 1999) and enantioselectivity (Jaeger and Reetz, 2000) for biocatalyst optimization, to change substrate specificity of enzymes for synthesis of novel compounds (Schmidt-Dannert et al., 2000) , and to increase tolerance to toxic products or solvents for improved process development (Selifonova et al., 2001). The genes encoding the desired enzymes are generally cloned on multi-copy vectors. Several properties of the vectors such as copy number, temperature sensitivity, strength of regulatable promoters can also be modulated to facilitate biocatalysis and metabolic engineering.

Plasmid replication is generally controlled in the cell, so that the copy number of a given plasmid stays within a narrow range in a given host under defined growth conditions. Host chromosomal mutations (Ederth et al., 2002; Lopilato et al., 1986) and *cis*- and *trans*-acting elements on plasmids (Cesareni et al., 1982; Moser et al., 1984) were reported to alter the copy number of plasmids of ColE1 replicons. Amino acid substitutions at the *rep* gene increased copy number of plasmid pSC101 (Wadood et al., 1997). Mutations in the gene *trfA* encoding replication initiation protein produced increased copy number derivatives of broad-host range plasmid RK2 in *Escherichia coli* and distantly related bacteria (Fang et al., 1993).

Plasmid pBBR1 is a small broad-host range plasmid (Antoine and Locht, 1992) isolated from *Bordetella*

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doi:10.1016/j.ymben.2004.05.006

pertussis. It does not belong to the incompatibility groups of IncP, IncQ or IncW. Two open reading frames were identified on pBBR1: Rep, which was involved in plasmid replication; and Mob, which was involved in mobilization. The mechanism for pBBR1 replication is still largely unknown. Many derivatives of pBBR1 were constructed with addition of multiple cloning sites (Kovach et al., 1994), antibiotic resistance markers (Kovach et al., 1995), reporter genes (Ramos et al., 2002), and regulated promoters (Lefebre and Valvano, 2002; Sukchawalit et al., 1999). They have been used in a variety of applications including development of genetic system for bacteria (Coppi et al., 2001; Su et al., 2001), synthesis of novel polyhydroxy alkanoates (Ewering et al., 2002), production of biocatalysts for biotransformation (Overhage et al., 2002), over-expression of protective antigen to enhance vaccine efficacy (Vemulapalli et al., 2000). For many of the applications, increased copy number of the pBBR1-based plasmids would be beneficial since the titer of the products were likely to be increased.

In this study, the replication control region of a pBBR1-based broad host range plasmid was targeted for mutagenesis to increase the plasmid copy number. Mutant plasmid derivatives were isolated that contained single or multiple mutations in the *rep* gene and/or the flanking regions. The copy number of these plasmid derivatives increased 3–7 fold comparing to that of the parent plasmid. The increased copy number plasmids were used to co-express an isoprenoid pathway gene to engineer high titers of carotenoid production in *E. coli*. The utility of the plasmid derivatives was expanded to non-*E. coli* hosts.

2. Materials and methods

2.1. Construction of β-carotene reporter plasmids

Pantoea stewartii ATCC8199 contains a natural gene cluster *crtEXYIBZ* for carotenoid synthesis. The useful genes *crtE* and *crtYIB* for β-carotene synthesis was joined together by PCR. The *crtE* gene was amplified using forward primer pBHRcrt_1F (5′-GAATTC GCCCTTGACGGTCT-3′) and reverse primer pBHRcrt_1R (5′-CGGTTGCATAATCCTGCCCACT-CAATTGTTAACTGACGGCAGCGAGTTTT-3′). The *crtYIB* gene fragment was amplified using forward primer pBHRcrt_2F (5′AAAACTCGCTGCCGTCAGT TAACAATTGAGTGGGCAGGATTATGCAACCG-3′) and reverse primer pBHRcrt_2R (5′-GGTACCTA GATCGGGCGCTGCCAGA-3′). Underlined are restriction sites for *EcoR* I, or *Kpn* I. The *Kpn* I site was introduced to facilitate construction of pDCQ318 later. The two PCR products were gel purified and joined together by another PCR using primer pBHRcrt_1F and pBHRcrt_2R. The final 4511 bp PCR product was cloned into pTrcHis2-TOPO vector (Invitrogen, Carlsbad, CA) in the forward orientation, resulting in plasmid pDCQ300. The ~4.5 kb *EcoR* I fragment of pDCQ300 containing the *crtEYIB* gene cluster was ligated to the unique *EcoR* I site in pBHR1 vector (MoBiTec GmbH, Goettingen, Germany). In the resulting construct, pDCQ301, the *crtEYIB* genes were under the control of the chloramphenicol resistant gene promoter on the pBHR1 vector.

Another reporter plasmid pDCQ318 was constructed from pDCQ301 to shorten the 3′ flanking region of the *rep* gene that would be subjected to error-prone PCR mutagenesis. The *rep* gene and its flanking regions of pDCQ301 was PCR amplified using primer (5′-AGATTGTCGCACCTGATTGC-3′) and (5′-CGGGGTACCGAATTCTACAGCCGATAGTCTG-GAACAGC-3′). The underlined is an engineered *Kpn* I site. The 1388 bp PCR product was digested with *Xho* I and *Kpn* I, and ligated into *Xho* I and *Kpn* I sites of pDCQ301, resulting in pDCQ318. The original *rep* gene region was replaced with the new one, which removed approximately 850 bp from the 3′ flanking region of the *rep* gene.

2.2. Error-prone PCR mutagenesis

Error prone PCR mutagenesis was performed on a 1461 bp fragment from pDCQ318 that contained the pBHR1 *rep* gene. PCR reaction was performed with AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA) in GeneAmp® 10X PCR Buffer II containing $MgCl_2$ (250 μM), dNTPs (200 μM of each), primers (pBHR1rep_F: 5′-ATCGATAGATTGTCG-CACCTGATTG-3′ and 301rep_R2: 5′-CATCCGGT-CAGGCAGTTACT-3′), and pDCQ318 DNA (10 ng). In addition, each reaction contained 50, 75, 100, 125, 150 or 200 μM $MnCl_2$. The PCR conditions were as follows: 5 min at 95 °C; then 35 cycles at 92 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min; followed by additional 10 min at 72 °C. The purified PCR products from reactions containing different amount $MnCl_2$ were combined and digested with *Kpn*I and *Xho* I. The 1300 bp digested error prone PCR product was gel purified and ligated to *Kpn* I/*Xho* I sites of pDCQ318 to replace the wild-type *rep* gene. The ligated DNA was electroporated into *E. coli* XL1-blue MRF' cells (Stratagene, La Jolla, CA). The transformed cells were spread on LB agar containing 50 μg/ml kanamycin and incubated at 30 °C for 3–4 days.

2.3. β-carotene assay

Each strain containing the mutant plasmid was inoculated into 5 ml LB medium with 50 μg/ml kanamycin. Cells were grown at 30 °C with shaking for 2–3

days. XL1-blue MRF' containing pDCQ301 or pDCQ318 cells were used as controls. Cell densities were recorded by measuring OD_{600}. Cells were spun down for 15 min at 4000*g*. Carotenoids were extracted from cell pellets in 0.5 ml acetone for 10 min at room temperature. β-carotene has characteristic absorption spectra of (455 nm, 480 nm in acetone) with the absorption maxima at 455 nm. The relative amount of β-carotene produced was recorded by measuring OD_{455} and normalized by cell density. The OD_{455}/OD_{600} value for each mutant was compared with that of the controls.

2.4. Real-time PCR

Crude lysate samples containing various plasmids were prepared for copy number determination by real-time PCR. Each *E. coli* strain was grown in 10 ml LB medium with 50 μg/ml kanamycin at 37 °C for 17 h. Several plasmids were also introduced into *Agrobacterium tumefaciens* C58 by electroporation. *Agrobacterium* strains containing the plasmid derivatives were grown in 10 ml LB medium with 50 μg/ml kanamycin at 30 °C for 32 h. Cell pellet from 1 ml culture was resuspended in 1 ml dH_2O. Approximately 0.25 ml 0.1 mm ZirConia silica beads (Biospec Products Inc., Bartlesville, OK) was added into the cell suspension and the mixture was beaten for 2 min in the BeadBeater (Biospec Products Inc.) to lyse the cells. The crude lysate was then spun for 2 min at 12,000*g* in a micro-centrifuge. Aliquots of 100 μl supernatant was heated at 99 °C for 10 min to inactivate DNase activity. Seven 1:10 serial dilutions were prepared for each crude DNA sample. A sample contained no DNA was also included as negative control. PCR primers were designed using the default settings in Primer Express v 2.0 software from Applied Biosystems. Forward primer 5′-CCGCGCCTGAGCCTAAT-3′ and reverse primer 5′-CGGCAAAGACATCAATCAGGAT-3′ were designed to amplify a 65 bp region at the 3′ end of the *crtE* gene on target plasmid DNA. A 62 bp region of the *E. coli* 16S rRNA gene was amplified using forward primer 5′-CCAGCAGCCGCGGTAAT-3′ and reverse primer 5′-TGCGCTTTACGCCCAGTAAT-3′. A 60 bp region of the 16S rRNA gene of *A. tumefaciens* C58 (GenBank accession number AE009348) was amplified using forward primer 5′-TTTGCTCCCCACGCTTTC-3′ and reverse primer 5′-TGGCGAAGGCGGCTTA-3′. Real-time PCR was performed using SYBR Green labeling method and monitored on an ABI 7900 Sequence Detection System instrument according to the manufacturer's instructions. The PCR reaction (20 μl) was set up using the following reagents: 10 μl ABI 2XSYBR Green Master Mix, 0.2 μl each of the forward and reverse primers (100 μM), 8.6 μl water and 1 μl of diluted DNA sample. The thermal cycling conditions used were as follows: 10 min at 95°C followed by 40 cycles of 95 °C, 15 s and 60 °C, 1 min. All reactions were run in triplicate.

2.5. Cloning of dxs expression plasmid

The *dxs* gene from *Rhodococcus erythropolis* AN12 was amplified using an upstream primer 5′-ATGGGTGTTCTTGCCCGCATTC-3′ and a downstream primer 5′-TCAGCCTTGCGCGACTTCGCCC-3′. The PCR product was cloned into the pTrcHis2-TOPO vector. The construct pDCQ110 contained *dxs* gene cloned in the forward orientation under the trc promoter.

3. Results and discussion

3.1. Isolation of mutant derivatives of plasmid pDCQ318 with increased β-carotene production

Broad host range plasmid pBHR1 (Szpirer et al., 2001) is a derivative of pBBR1 with the same replication control region. The carotenoid synthesis genes *crtEYIB* encode enzymes for synthesis of β-carotene, which was used as a reporter for mutant screen in this study. Two plasmids were constructed: pDCQ301 and pDCQ318 (Fig. 1). pDCQ301 contains the *crtEYIB* gene cluster cloned into the *EcoR*I site of the broad host range vector pBHR1. pDCQ318 contains a deletion of the downstream region of the *rep* gene from pDCQ301, which shortens the region that would be subjected to error-prone PCR mutagenesis. A library of over 10,000 clones was generated by error-prone PCR of the replication region on pDCQ318, among which three-dozen clones were isolated that showed darker yellow color. These clones were streaked twice and shown to be relatively stable. β-carotene assay was performed with the three-dozen mutants and the result of ten mutants that showed the highest increase in β-carotene production was shown in Fig. 2. These ten mutants produced 2–3 fold as much of β-carotene as that of the parent. The colonies of the mutant strains generally appeared smaller. Their slower growth is likely due to increased β-carotene production and/or increased plasmid burden. Several tiny colonies with intense yellow color were also observed, however, they are unstable after restreaking.

Some colonies of lighter yellow color were also present in the mutant library. They probably contained mutant plasmids with decreased plasmid copy number. Plasmids with decreased copy number are also useful for metabolic engineering, such as for tuning down expression of toxic genes in heterologous hosts (Podkovyrov and Larson, 1995).

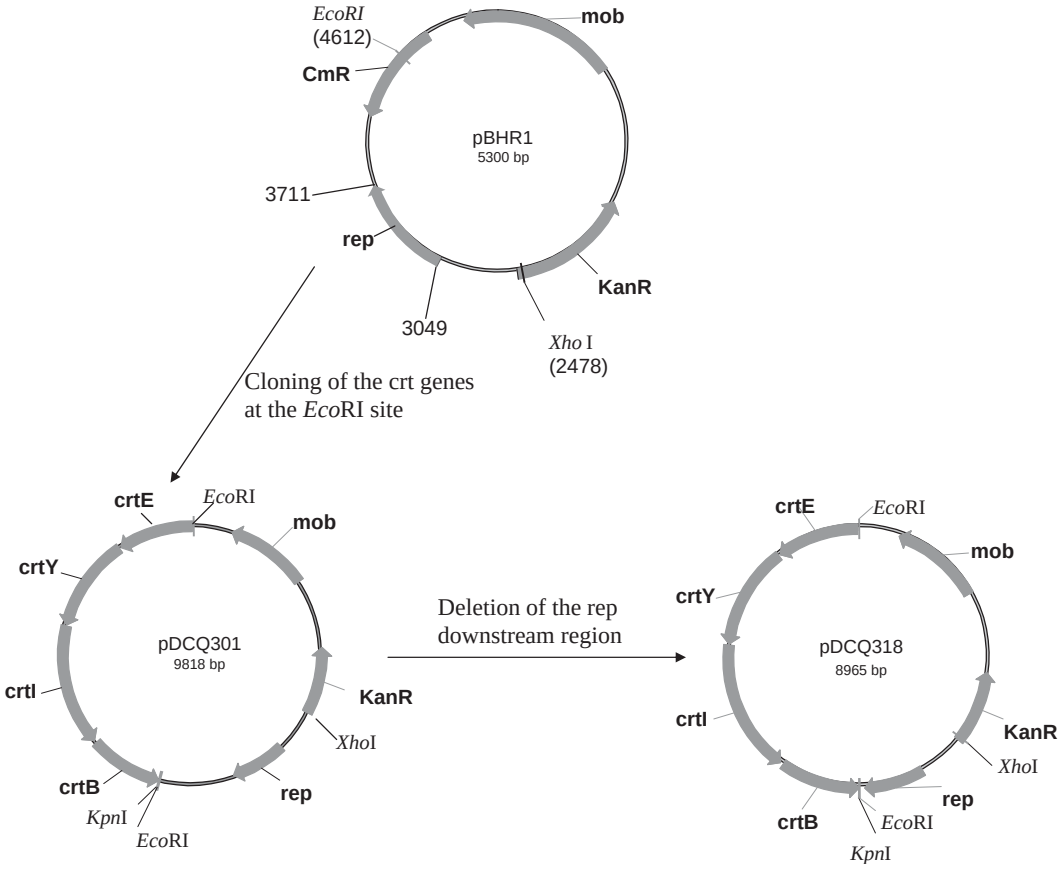

Fig. 1. Construction of pBHR1 based reporter plasmids pDCQ301 and pDCQ318.

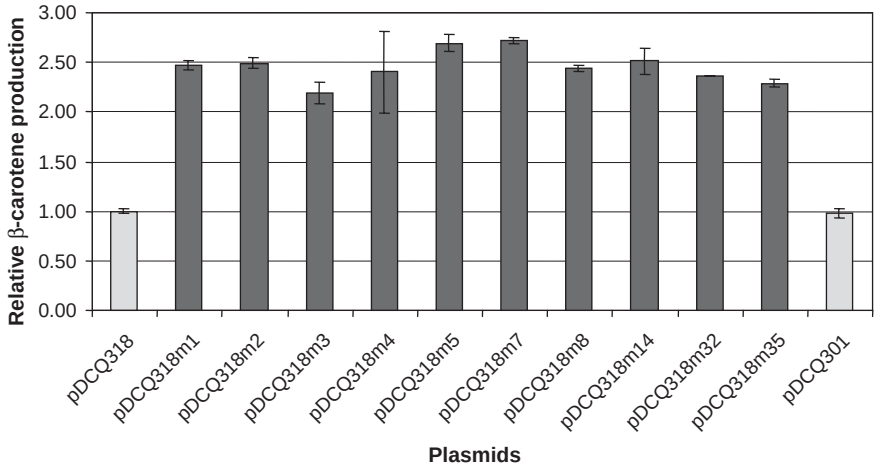

Fig. 2. Assay of β-carotene in *E. coli* containing various carotenoid reporter plasmids. The β-carotene production was normalized to cell density in each strain. The value was then compared with that of the parent strain containing pDCQ318.

3.2. Identification of mutations in the replication control region

The 10 mutants that showed the highest increase in β-carotene production were selected for further characterization. The replication control region of the plasmids were sequenced and aligned with the corresponding wild type pBHR1 sequence (2478–3765 nucleotides of pBHR1, GenBank Accession No. Y14439). The parent plasmid pDCQ318 had an insertion of cytosine at nucleotide 3030 comparing to pBHR1. This C3030 insertion, which was 19 bp upstream of the start codon of the *rep* gene, was generated by PCR during construction of pDCQ318. Since amount of β-carotene

synthesis from pDCQ318 is similar to that from pDCQ301 containing the wild type *rep* region, this mutation did not cause increased β-carotene production. All the pDCQ318 mutants inherited this mutation from pDCQ318. The additional mutations generated by error-prone PCR mutagenesis in pDCQ318 mutant plasmids are summarized in Fig. 3. Because of the wide range of $MnCl_2$ concentrations used for mutagenesis, the number of mutations obtained in each mutant varied from 1 to 7.

The 10 different mutants can be categorized into 4 groups. The first group of mutants includes M2, M5, M7 and M14. They all contained a mutation of C to T at nucleotide 3347 (C3347T), which resulted in serine to leucine change at codon 100 of the *rep* gene (S100L). M2 is a mutant containing the single C3347T mutation. M5, M7 and M14 contained other mutations in addition to C3347T. The phenotype of M2 suggested that single C3347T mutation is sufficient for the increased β-carotene production. The second group of mutants includes M4, M8, M32 and M35. They all contained a mutation of C to T at nucleotide 3003 (C3003T), which is 46 bp upstream of the start codon of the *rep* gene. The C3003 T mutation is not in the putative –10, −35 promoter region or the upstream direct repeats region (Antoine and Locht, 1992). It could still affect transcription of the *rep* gene. The phenotype of M8 with single C3003T mutation suggested that C3003T mutation is sufficient for the increased β-carotene production. The effect of other additional mutations is not known. M1 and M3 constitute the other two mutant groups. M1 contained two mutations within the *rep* gene. Substitutions T3262G and T3344C resulted in amino acid changes L72V and V101A. M3 contained two different mutations within the *rep* gene. Substitutions C3269T and T3604C resulted in amino acid changes T74M and W186R. M3 also contained three additional mutations further upstream of the directed repeats in the 5′ flanking region of the *rep*. It is not clear which specific mutation or combination of mutations caused increased β-carotene production in M1 and M3. Likely V101A mutation had some effect since it is next to the S100L mutation site, which itself was sufficient for increased β-carotene production. Deletion of cytosine at nucleotide 2634 upstream of *rep* appeared in four mutants (M3. M5, M7 and M32), however, its effect as a single mutation is not known.

3.3. Determination of relative copy number of plasmid pDCQ318 mutant derivatives in E. coli

Real-time PCR has been widely used to study gene expression by quantifying specific message RNA in the cell (Bustin, 2000). It can also be used to quantify

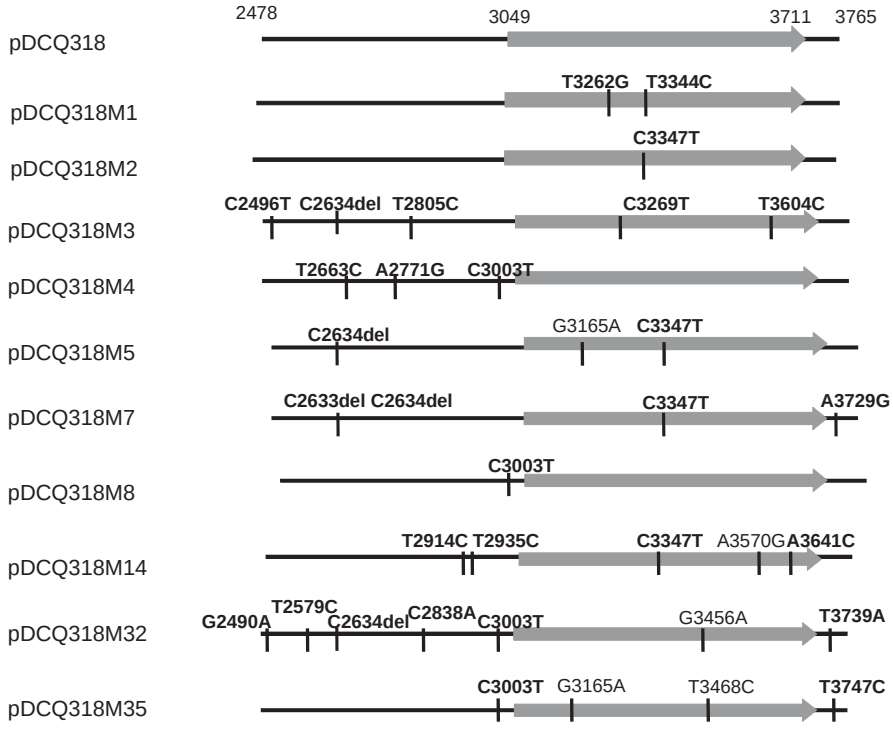

Fig. 3. Mapping of mutations in pDCQ318 and derivatives. The replication control region (2478–3765 bp) for each of the plasmid is shown, with the blocked arrow representing the location of the *rep* gene (3049–3711 bp). Mutations were numbered using the corresponding base pair positions in pBHR1. The nucleotides before the numbers indicate the wild type sequences. The nucleotides after the numbers indicate the mutated sequences. "del" after the numbers indicate deletion of a nucleotide at that position. The non-bolded mutations within the *rep* gene indicate silent mutations. The insertion of cytosine at 3030 bp present in pDCQ318 and all the mutant derivatives was not included in this figure.

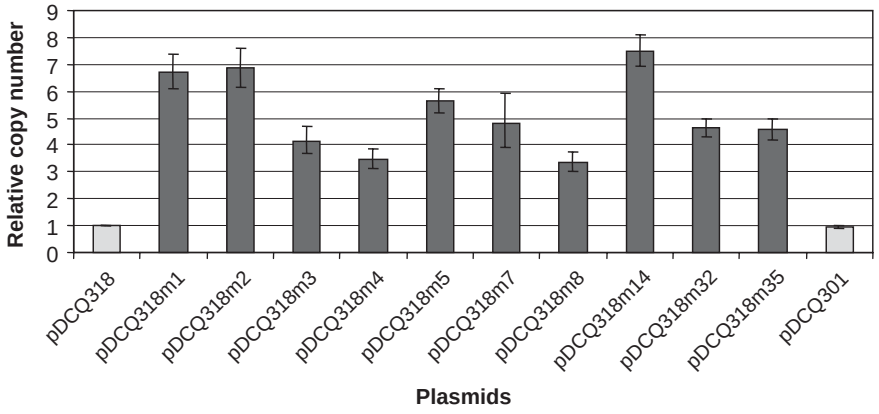

Fig. 4. Quantitation of relative copy number of pDCQ318 derivatives in *E. coli* by real-time PCR. ΔΔCt method was used for quantitation as described in Results and Discussion. *E. coli* 16S rRNA gene was used for normalization of DNA concentration for each sample. Plasmid pDCQ318 was used as the reference sample.

specific DNA in the cell. Many of these applications include measuring DNA copy number, transgene copy number, and viral titers (Ginzinger, 2002). We used real-time PCR to determine relative copy numbers of pDCQ318 mutant derivatives using pDCQ318 as the reference sample. 16S rRNA was used to normalize the quantitation of each sample for differences of total DNA amount added to each reaction. The normalized threshold cycle number is referred to as ΔCt. The ΔCt of each pDCQ318 mutant compared to that of the pDCQ318 reference is referred to as ΔΔCt. The ΔΔCt values were then converted to absolute values by utilizing the formula $2^{-\Delta\Delta Ct}$. These values refer to the fold increase in the copy number of pDCQ318 mutant plasmids comparing to the pDCQ318 parental plasmid. The PCR efficiencies of the 16S rRNA gene and the target gene in each sample are close to 100%. This validates the use of the ΔΔCt method for quantitation of relative copy number of plasmid DNA.

The copy numbers of the pDCQ318 mutant plasmids increased 3–7 fold in *E. coli* (Fig. 4) comparing to that of the parental plasmid pDCQ318, which did not change from that of pDCQ301. The insertion of C at nucleotide 3030 introduced during construction of pDCQ318 appeared to be a random mutation that did not affect the plasmid copy number. The increased copy numbers of the pDCQ318 mutant plasmids were caused by other mutations (Fig. 3) generated by error-prone PCR mutagenesis.

The increased β-carotene production in the mutant strains are most likely due to increased copy number of the plasmids carrying β-carotene synthesis genes. However, the fold of β-carotene production did not correlate exactly with the fold of copy number increase. The dynamic range of β-carotene production was smaller than that of the relative copy number increase. This is likely due to the limiting isoprenoid precursors for carotenoid synthesis. Several bottlenecks in the isoprenoid pathway (*dxs*, *idi*) have been reported to limit the flux for carotenoid biosynthesis (Albrecht et al., 1999). Therefore, amount of carotenoid production would not be expected to be a linear proportion to the copies of the carotenoid synthesis genes.

3.4. Co-expression of β-carotene synthesis genes with dxs in E. coli

To demonstrate utility of the increased copy number plasmids for metabolic engineering, two mutant plasmids containing the β-carotene synthesis genes were co-expressed with an isoprenoid pathway gene (*dxs*) on a compatible plasmid. The gene *dxs* encodes the 1-deoxyxylulose-5-phosphate synthase that catalyzes the first step of the isoprenoid pathway for carotenoid synthesis. It was reported that overexpression of *dxs* in *E. coli* increased the flux of the isoprenoid pathway (Harker and Bramley, 1999). The *dxs* gene from *Rhodococcus erythropolis* (Kostichka et al., 2003) was expressed in *E. coli* containing the β-carotene synthesis plasmids pDCQ301 or pDCQ318. It increased β-carotene production approximately 2.5 fold. When it was co-expressed with mutant plasmids pDCQ318M1 or pDCQ318M14, over 3.5 fold increase of β-carotene production was observed (Fig. 5). The combination of the *dxs* overexpression and the plasmid copy number increase was slightly synergistic, although not as much as one might have expected. Another step of the isoprenoid pathway (such as the Dxr or the Idi step) might have become the new bottleneck after relieving of the first bottleneck of Dxs. It was reported that multiple bottlenecks exist in the isoprenoid pathway (Albrecht et al., 1999) and overexpression of multiple genes in the isoprenoid pathway had additive effect on increase of carotenoid production. It is likely that the isoprenoids are still limiting after expression of *dxs*, which explains

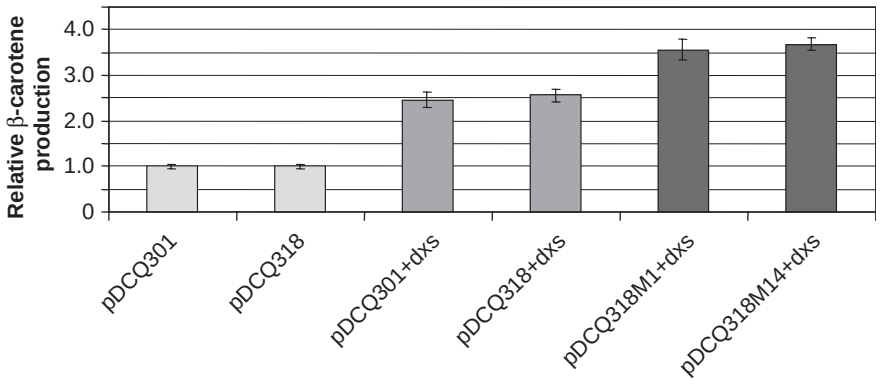

Fig. 5. Co-expression of β-carotene synthesis genes with *dxs* in *E. coli*. The β-carotene synthesis genes were expressed on pDCQ301, pDCQ318 or the two mutant derivatives (M1 and M14) of pDCQ318. The *dxs* gene was expressed on another compatible plasmid pDCQ110. The β-carotene production was normalized to cell density in each strain. The value was then compared with that of the parent strain containing pDCQ318.

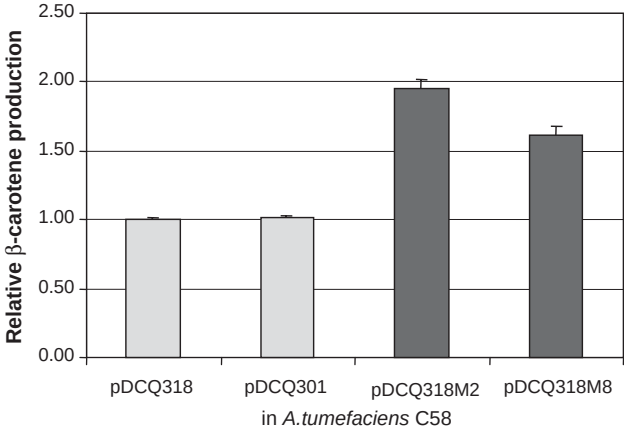

Fig. 6. Assay of β-carotene in *A. tumefaciens* containing various plasmids. The β-carotene production was normalized to cell density in each strain. The value was then compared with that of the parent strain containing pDCQ318.

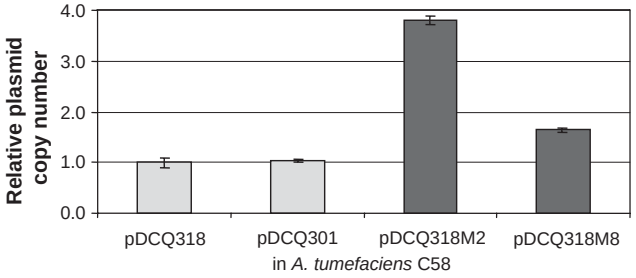

Fig. 7. Determination of relative copy number of pDCQ318 derivatives M2 and M8 in *A. tumefaciens* by real-time PCR. $\Delta\Delta$Ct method was used for quantitation as described in Results and Discussion. 16S rRNA gene of *A. tumefaciens* was used for normalization of DNA concentration for each sample. Plasmid pDCQ318 was used as the reference sample.

the slight synergistic effect with the plasmid copy number increase.

3.5. Application of the copy number plasmid derivatives in a non-E. coli host

Since use of the pBBR-based broad host range plasmids has been reported in many bacterial hosts, we are interested in testing the application of the copy number plasmid derivations in a non-*E. coli* host. The plasmid derivatives pDCQ318M2 and pDCQ318M8 containing single mutation, together with the control plasmids pDCQ318 and pDCQ301, were introduced into *A. tumefaciens* C58 strain. The *Agrobacterium* strains containing the plasmids appeared yellow comparing to the colorless parent strain without the plasmids. This suggested that pBBR-based plasmids replicate in *Agrobacterium*. The colonies containing M2 or M8 appeared smaller and slightly darker-colored comparing to those containing the control plasmids. β-carotene assay (Fig. 6) indicated that 1.6–2 fold as much of β-carotene was produced in the strains containing the plasmid derivatives comparing to that of the control plasmids. The copy number of the plasmid derivatives in the *Agrobacterium* strain (Fig. 7) was 1.7–3.8 fold as much as that of the control plasmids. The copy number of M2 was higher than that of M8 in *Agrobacterium* as was the case in *E. coli*, although the exact numbers are different in the two hosts. It was also observed in *Agrobacterium* that the fold of carotenoid production was smaller than the fold of plasmid copy number increase, likely due to limiting isoprenoid precursors.

Acknowledgments

We are grateful to Mike Madden for his expert assistance with real-time PCR analysis.

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