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Isolation of chromosomal mutations that affect carotenoid production in *Escherichia coli*: mutations alter copy number of ColE1-type plasmids

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Abstract

Chromosomal mutants were isolated in *Escherichia coli* that altered carotenoid production from transformed carotenoid biosynthesis genes on a pACYC-derived plasmid (pPCB15). The mutations were mapped by sequencing. One group of mutations appeared to affect the cell metabolism without changing the copy number of the carotenoid synthesis plasmid. The other group of mutations either increased or decreased the copy number of the pPCB15 plasmid as determined by real-time PCR. The copy number change in most mutants was likely specific for ColE1-type plasmids for which copy number is controlled by a small antisense RNA. This collection of host strains would be useful for fine tuning expression of proteins and adjusting production of desired molecules without recloning to different vectors.

Keywords: Transposon mutagenesis; Plasmid copy number; Carotenoid biosynthesis

1. Introduction

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. The mechanism for ColE1-type plasmid replication is well-studied and is controlled by antisense regulation [1,2]. ColE1-type plasmids include plasmids with a pMB1 (e.g. pBR322) or p15A (e.g. pACYC184) origin of replication. In these plasmids, replication is mediated by formation of the hybrid between the primer transcript RNA II and the template DNA near the origin of replication. RNA I is an antisense RNA that binds to RNA II and inhibits hybrid formation. In plasmids containing the intact pMB1 replication origin, the plasmid encoded protein Rop enhances the inhibitory effect of RNA I by enhancing the binding of RNA I to RNA II [3]. Copy number mutants were isolated that contained mutations in the Rop [4] or the RNA I [5] or RNA II [6] coding region on the plasmid. Host chromosomal mutations such as *pcnB*, *relA* and *rpoC* were also reported to alter the copy number of ColE1-type plasmids. The *pcnB* [7] or *rpoC* [8] mutations were specific for plasmids of ColE1-type origin, whereas the *relA* mutations could also amplify other plasmids such as pSC101-derived replicons and λ plasmids [9,10].

Carotenoids are a diverse group of natural pigments found in microorganisms and plants, which are

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currently used as food colorants, nutritional supplements and pharmaceuticals [11]. Non-carotenogenic organisms such as *E. coli* can synthesize carotenoids by extending their isoprenoid pathway through recombinantly introduced carotenoid synthesis genes. The non-mevalonate isoprenoid pathway of *E. coli* is not fully elucidated and the regulation of carotenoid synthesis is largely unknown. Nevertheless, considerable effort exists to increase carotenoid production in *E. coli* by metabolic engineering [12–15]. We took a random approach to isolate *E. coli* transposon mutants that altered carotenoid production. Some mutations affected copy number of the carotenoid synthesis plasmid.

2. Materials and methods

2.1. Construction of β-carotene reporter plasmids

The *crtEXYIB* gene cluster for carotenoid synthesis was amplified from *Pantoea stewartii* ATCC8199 [16] and cloned into *Sma*I digested pSU18 vector, a plasmid carrying the p15A replicon [17]. Following transformation into *E. coli*, colonies containing the resulting plasmid pPCB15 appeared to be bright yellow and were shown to produce β-carotene.

2.2. Transposon mutagenesis

E. coli MG1655 containing pPCB15 was used for transposon mutagenesis. Mutagenesis was performed using EZ:TN <KAN-2>Tnp Transposome kit (Epicentre Technologies, Madison, WI) according to the manufacturer's instructions. Mutants were plated onto LB-Noble Agar (Difco Laboratories, Detroit, MI) plates containing 25 μg/ml kanamycin and 25 μg/ml chloramphenicol, and incubated at 37 °C overnight. Approximately 20,000 Kan^R mutant colonies were visually examined for deeper or lighter color development after being kept at room temperature for several days.

2.3. Mapping of the transposon insertion sites

The transposon insertion site in each mutant was identified by modified single-primer PCR [18] and sequencing of the PCR product. A 100 μl overnight culture was heated at 99 °C for 10 min and cell debris was removed by centrifugation. A 1 μl volume of the supernatant was used in a 50 μl PCR reaction using either Tn5PCRF (5′-GCTGAGTTGAAGGATCAGATC-3′) or Tn5PCRR (5′-CGAGCAAGACGTTTCCCGTTG-3′) primer. PCR was carried out as follows: 5 min at 95 °C; 20 cycles of 92 °C for 30 s, 60 °C for 30 s, 72 °C for 3 min; 30 cycles of 92 °C for 30 s, 40 °C for 30 s, 72 °C for 2 min; 30 cycles of 92 °C for 30 s, 60 °C for 30 s, 72 °C for 2 min. The PCR products were purified and sequenced using FP-1 or RP-1 primers provided in the Transposome kit. The chromosomal insertion site of the transposon was identified as the junction between the Tn*5* transposon and MG1655 chromosome DNA by aligning the sequence obtained from each mutant with the *E. coli* genomic sequence (GenBank Accession No. U00096).

2.4. β-carotene assay

MG1655 and each mutant containing the β-carotene synthesis plasmid pPCB15 was inoculated into 5 ml LB medium with 25 μg/ml chloramphenicol. Cells were grown at 30 °C with shaking for one day. Cell densities were measured spectrophotometrically as OD_{600}. Cells were spun down and carotenoids were extracted from cell pellets with acetone. β-carotene has characteristic absorption spectra at 455 and 480 nm with the absorption maximum at 455 nm. The relative amount of β-carotene produced was recorded by measuring OD_{455} and normalized with regard to cell density. The OD_{455}/OD_{600} value for each mutant was compared with that of the parent strain.

2.5. Estimation of plasmid copy number by agarose gel staining

Cells were grown in LB containing chloramphenicol (25 μg/ml) with shaking overnight. Cell densities were measured by OD_{600}. Plasmid DNA was isolated from the same amount of cells (normalized by OD_{600}) from different strains. Plasmid DNA was linearized by *Eco*RI digest, and 1, 2 and 4 μl of digested DNA were loaded on an agarose gel. The change of plasmid copy number was estimated by comparing the intensities of the linearized plasmid band on the agarose gel after ethidium bromide staining.

2.6. Real-time PCR

Each *E. coli* strain containing pPCB15 was grown in LB medium with 25 μg/ml chloramphenicol at 30 °C for 24 h. Crude lysate samples were prepared for copy number determination by real-time PCR as described previously [19]. The 65 bp region at the 3′ end of the *crtE* gene was used as the amplicon for target plasmid DNA. The 62 bp region of the *E. coli* 16S rRNA gene was amplified for normalization. Real-time PCR was performed using the SYBR Green labeling method and monitored on an ABI 7900 Sequence Detection System instrument according to the manufacturer's instructions. All reactions were run in triplicate. The PCR efficiencies for both the pPCB15 and the 16S rRNA primers were close to 100%, which validated the method for quantitation of relative copy number of plasmid DNA. The normalized threshold cycle number is

referred to as ΔCt. The ΔCt of each mutant compared to that of the parent is referred to as ΔΔCt. The ΔΔCt values are converted to absolute values by utilizing the formula $2^{-\Delta\Delta Ct}$ (as per "User Bulletin #2: Relative Quantitation of Gene Expression", ABI, 10/2001). These values refer to the fold increase in the copy number of the plasmid in the mutants compared to that in the parent strain.

2.7. Luciferase assay

Cells containing pTV200 were grown in LB with 10 μg/ml of tetracycline at 37 °C with shaking overnight. Triplicates of each culture were pipetted into a 96-well plate and luciferase activity was measured using HTS 7000 Plus BioAssay Reader (Perkin–Elmer, Norwalk, CT). Cell density of the samples (OD_{600}) in each well was also measured for normalization using the BioAssay Reader.

3. Results and discussion

3.1. Isolation of transposon-generated mutants that affected β-carotene production

E. coli MG1655 is non-carotenogenic and synthesizes only the farnesyl pyrophosphate precursor via the isoprenoid pathway. When the *crtEXYIB* gene cluster from *P. stewartii* [16] was introduced into *E. coli* on plasmid pPCB15, β-carotene was synthesized (Fig. 1) and the cells became yellow. *E. coli* chromosomal mutations which increase or decrease carotenoid production should result in darker or lighter color colonies. *E. coli* MG1655 containing pPCB15 was subject to transposon mutagenesis. Approximately 20,000 mutant colonies were visually examined, among which eight distinctly darker yellow clones were isolated and six distinctly lighter yellow or white clones were isolated. These clones were streaked twice and shown to be relatively stable. The colonies of several darker yellow mutants (Y1, Y8, Y12, Y15 and Y17) appeared small, among which Y8 and Y15 were the smallest. β-carotene assay was performed with these mutants and the results are shown in the top portion of Fig. 2. The darker yellow mutants produced approximately 2–3-fold as much of β-carotene as the parent control. The lighter yellow mutants (W5 and W6) produced approximately 20–40% of β-carotene compared to the parent control. The white mutants (W1, W3, W4 and W16) produced less than 5% of the amount of β-carotene formed by the parent control.

3.2. Identification of transposon insertion sites

The transposon insertion site in each mutant was identified directly by PCR and sequencing of the mutant strain as described in Section 2. The chromosomal insertion sites in the mutants were later confirmed by PCR using pairs of primers designed based on the transposon end sequences and the specific sequences around the insertion sites. Fig. 3 summarizes the chromosomal insertion sites of the mutants that affected carotenoid production. The genes flanking each of the transposon insertions are also shown. Several of the genes (*thrS*, *rpoC*, *mreC*, *rhoL*, *hscB*) were part of characterized or predicted operons.

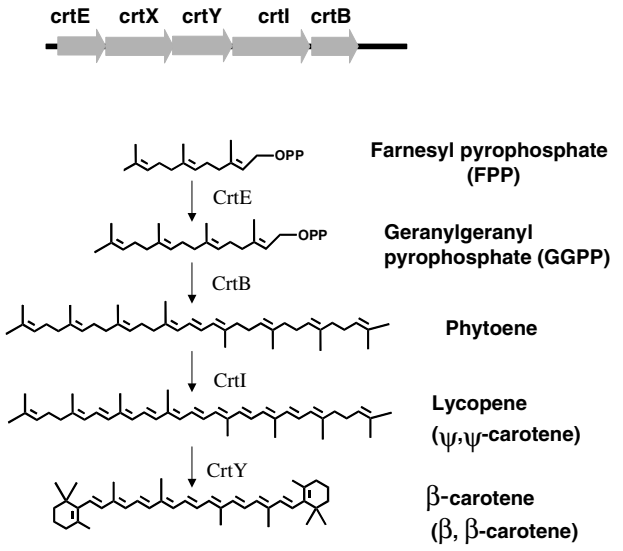

Fig. 1. Gene structure and the biochemical pathway for β-carotene synthesis. The *crtEXYIB* genes expressed on pPCB15 plasmid were from *P. stewartii* ATCC8199. The arrows indicate the direction of transcription of the genes. CrtE: geranylgeranyl pyrophosphate synthase; CrtB: phytoene synthase; CrtI: phytoene desaturase; CrtY: lycopene β-cyclase. CrtX catalyzes a reaction beyond β-carotene synthesis and is not shown in this figure.

Two white mutants W1 and W16 not included in Fig. 3 contained a transposon insertion in the *crtI* or *crtE* gene on the pPCB15 plasmid. The *crtI* gene encoding phytoene dehydrogenase and the *crtE* gene encoding geranylgeranyl pyrophosphate synthase are part of the *crtEXYIB* gene cluster responsible for β-carotene synthesis. It was expected that transposon insertion in the *crtI* or *crtE* gene would result in white mutants, which validated the mutant isolation and screening method.

White mutant W3 had a transposon insertion in the *cya* gene encoding adenylate cyclase. It had been reported [20,21] that accumulation of carotenoids in *E. coli* requires cyclic AMP and is repressed by glucose. It was likely that carotenoid production in W3 was subjected to catabolic repression. Indeed, the yellow color was restored by addition of exogenous cAMP in the medium when growing W3. White mutant W4 had a transposon insertion in the *pcnB* gene, which controls the copy number of ColE1 plasmids [7,22]. It was likely that decreased carotenoid production in W4 was due to decreased copy number of the pPCB15 plasmid carrying

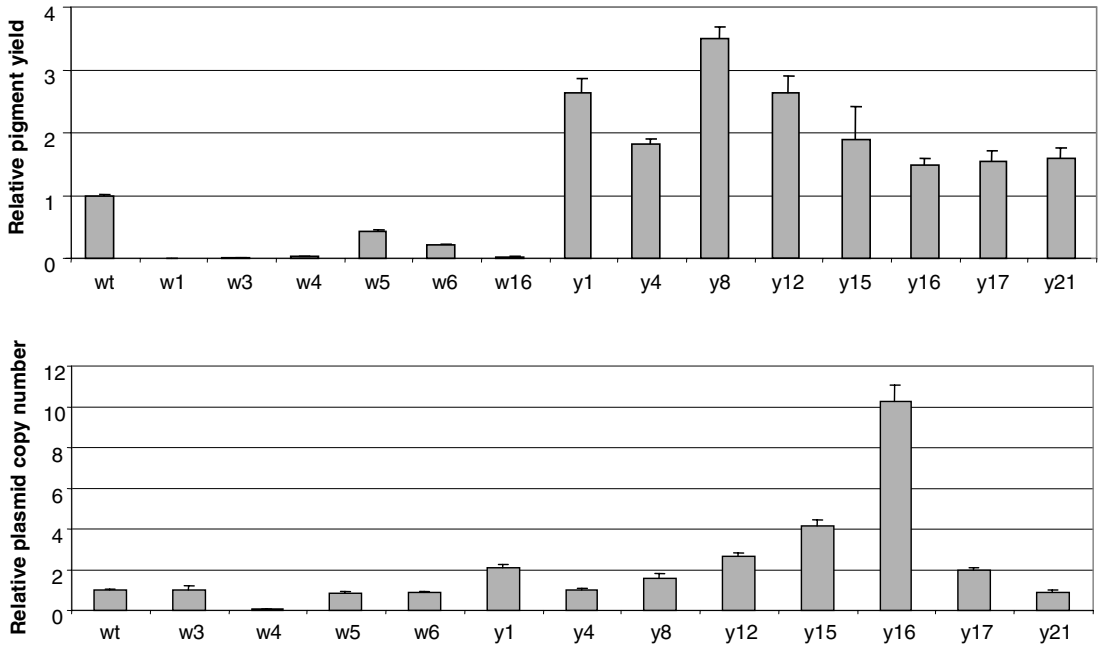

Fig. 2. Characterization of the *E. coli* transposon mutants. Top: Assay of β-carotene in *E. coli* mutants containing the carotenoid reporter plasmid pPCB15. The β-carotene yield was normalized to cell density (OD_{600}) in each strain. The value was then compared with that of the parent strain. The error-bars represent the standard deviations for three independent experiments. Bottom: Quantitation of relative copy number of pPCB15 in *E. coli* mutants by real-time PCR. ΔΔCt method was used for quantitation as described in Section 2. *E. coli* 16S rRNA gene was used for normalization of DNA concentration for each sample. Wild type MG1655 containing pPCB15 was used as the reference sample. The error-bars represent the standard deviations for the triplicates.

the *crt* gene cluster. This was shown later by quantitation of relative plasmid copy number. Lighter yellow mutants W5 and W6 contained a transposon insertion in the *mdh* gene for malate dehydrogenase or the *gltA* gene for citrate synthase. It had been reported that mutations in the operon related to the TCA cycle affected carotenoid production [23]. It was likely that insertion in *mdh* or *gltA* altered the carbon flux and affected the balance between the two precursors, glyceraldehyde-3-phosphate and pyruvate, which had been shown to be a major limiting factor for carotenoid biosynthesis [13].

Transposon insertions in many yellow mutants affected genes involved in transcription, translation or RNA stability. The gene affected in Y1 (*thrS*) encodes threonyl-tRNA synthetase for synthesis of charged threonyl-tRNA [24]. The gene affected in Y4 (*deaD*) encodes RNA helicase that plays a role in translation [25]. The gene affected in Y8 (*rpsA*) encodes 30S ribosomal subunit protein S1 required for translation of most natural mRNAs in vivo [26]. The gene affected in Y12 (*rpoC*) encodes RNA polymerase β′ subunit required for transcription [27]. The gene affected in Y15 (*orn*) enodes oligoribonuclease required for RNA degradation [28]. The gene affected in Y16 (*mreC*) encodes a rod-shape determining protein responsible for the rod shape of *E. coli* cells [29]. The gene affected in Y17 (*rhoL*) encodes the rho operon leader peptide upstream of the gene for transcriptional termination factor Rho [30]. The gene affected in Y21 (*hscB*) encodes a heat shock cognate protein involved in assembly of iron–sulfur clusters [31]. Five of the affected genes, *thrS* [24], *rpsA* [26], *rpoC* [32], *orn* [28], *rhoL* [33], were previously reported to be essential for viability of *E. coli* cells. The transposon insertions we obtained in these five genes were very close to the carboxyl terminal end of the genes (Fig. 3) and most likely resulted in functional although truncated proteins. The colony size in these five mutants was consistently smaller than that of other mutants.

3.3. Determination of relative copy number of plasmid pPCB15 in E. coli mutants

It was possible that change of carotenoid production in some mutants was due to change of the copy number of the plasmid carrying the carotenoid synthesis genes. The relative copy number of plasmid pPCB15 was assessed initially using the agarose gel method. It showed that mutant W4 had much less plasmid DNA, whereas the rest of the white mutants had a comparable amount of plasmid DNA to the parent strain. Yellow mutants, Y1, Y8, Y12, Y15 and Y17, appeared to have more plasmid DNA than the parent, whereas Y4, Y16 and Y21 had a comparable amount. To obtain more accurate results, we used quantitative real-time PCR to determine relative copy numbers of pPCB15 in the mutant strains,

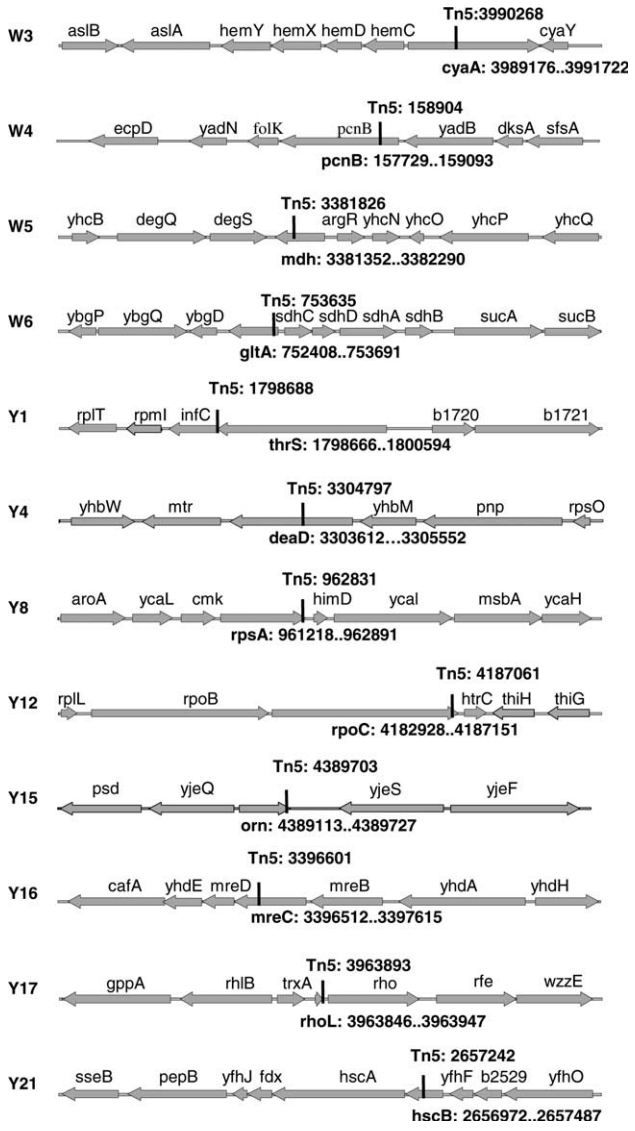

Fig. 3. Gene organization flanking the transposon insertion sites in the mutants. The genes are shown as block arrows with the gene names above or below. The direction of the arrows indicates the direction of transcription of the genes. The genes affected by the transposon and the transposon insertion sites are shown in bold. The numbers refer to the standard base pair (bp) numbers in the *E. coli* genome.

with the 16S rRNA gene being used to normalize the quantitation of each sample for differences of total amount of DNA added to each reaction.

The copy number of pPCB15 in *E. coli* mutants relative to the wild type is shown in the bottom portion of Fig. 2. Consistent with the agarose gel method, the plasmid copy number was found to be reduced more than 10-fold in W4, whereas it was similar to the wild type in W3, W5 and W6. The relative copy number determined in most of the yellow mutants also agreed with the agarose gel estimation. The copy number in Y1, Y8, Y12, Y15 and Y17 increased 2–4-fold, whereas it was similar to the wild type in Y4 and Y21. It is interesting that the strains with increased copy number (Y1, Y8, Y12, Y15 and Y17) all contained a transposon insertion near the carboxyl terminal of an essential gene. A difference in plasmid copy number was observed for Y16 between the agarose gel method and the real-time PCR method. The plasmid amount in Y16 normalized by cell density measured by OD_{600} showed no obvious difference from that of the wild type on an agarose gel (data not shown). However, real-time PCR normalized by 16S rDNA showed the highest copy number increase in Y16.

Hypotheses could be proposed to explain the altered plasmid copy number in the mutants. PcnB with polyadenylating activity is known to support replication by promoting decay of RNA I [34]. Decreased plasmid copy number in W4 was consistent with the phenotype of a previously reported *pcnB* mutant [7]. It had been suggested that uncharged tRNA molecules could interact with transcripts involved in regulation of ColE1-type replication [35]. It is possible that uncharged threonyl-tRNAs might bind and titrate RNA I due to observed sequence homology between regions of the threonyl-tRNAs and RNA I or RNA II, which could explain the increase of copy number in Y1 affected in threonyl-tRNA synthetase. RpoC mutants had previously been reported to have altered ColE1 plasmid copy number [8]. The two particular *rpoC* mutations, single amino acid substitution (G1161R) and a 42-amino acid deletion (Δ1149–1190) near the 3′-end of *rpoC*, were reported to reduce copy number of ColE1 plasmids. Y12 with the transposon insertion at codon 1378 of *rpoC* had the opposite effect of increasing the plasmid copy number. Possibly the *rpoC* mutation in Y12 affected expression of RNA I and RNA II promoters differently compared to the reported *rpoC* mutations [8], which resulted in an altered RNA I/RNA II ratio. Y15 with a transposon insertion in *orn* likely had a mutant oligoribonuclease, which affected stability of RNA I and RNA II that control plasmid copy number. Y17 had a transposon insertion in *rhoL*, which might affect the expression of *rho* downstream of *rhoL* in the same operon. It was reported that a *rho* mutation affected RNA I–RNA II interaction of ColE1 plasmids [36]. It is likely that the RNA-binding ability of Rho rather than its transcriptional termination function might have contributed to the plasmid copy number change in Y17. RpsA and DeaD are also RNA-binding proteins, however, whether they interact with ColE1-type plasmids is not known. The difference in their RNA-binding properties might be a likely explanation of the different effect on plasmid copy number in Y4 and Y8 mutants. Y16 with the insertion in *mreC* showed the highest increase (10-fold) of plasmid copy number as determined by real-time PCR. It was observed that the cells were near

round-shaped and almost twice the normal size (data not shown). The number of copies of plasmids partitioned into each cell was likely altered as the result of the cell morphology change. It is not clear if MreC has any direct role in plasmid segregation. The plasmid copy number increase in Y16 was not detected with the agarose gel method. The observed increase of β-carotene in Y16 was moderate (~1.5-fold). The change of cell shape and cell size possibly interfered with the cell density normalization by the spectrophotometric measurement for the β-carotene assay and the copy number estimation using the agarose gel method.

Five chromosomal mutants (W3, W5, W6, Y4 and Y21) affected β-carotene synthesis without obvious change of plasmid copy number. It is not surprising to obtain such a class of mutants, since many aspects of host cell metabolism and regulation can influence carotenoid synthesis. Mutations in these white mutants likely affected carotenoid regulation (W3) or precursor balancing (W5 and W6), which resulted in decrease of β-carotene synthesis. It is yet unclear how the mutations in Y4 or Y21 affected β-carotene synthesis.

3.4. Utility of the mutant strains affecting plasmid copy number

A collection of *E. coli* host strains was isolated in this study that altered copy number of pPCB15, a pACYC-derivative carrying carotenoid synthesis genes. This collection of host strains can be used to fine tune expression of proteins and adjust production of desired molecules without recloning to different vectors. Two representative strains were cured of pPCB15 and tested with other plasmids. Luciferase activity was tested with pTV200, a pACYC-derivative containing the *luxCDABE* reporter from *Photorhabdus luminescens* [37]. Luciferase activity decreased to 23% in W4 and increased 7.9-fold in Y15 compared to the wild type. To demonstrate if the utility for copy number modulation can be extended to other replicons, we transformed pACYC184 (p15A replicon, [38]), pBR328 (pMB1 replicon, [39]), pSC101 (pSC101 replicon, [40]), pBHR1 (pBBR1 replicon of broad host range plasmid, [41]), pMMB66 (RSF1010 replicon of IncQ broad host range plasmid, [42]) and pTJS75 (RK2 replicon of IncP broad host range plasmid, [43]) into the cured strains of W4 and Y15. Copy number of pACYC184 decreased in W4 and increased in Y15, which was consistent with other pACYC-derived plasmids used in this study. Similarly, the copy number of pBR328 was also observed to decrease in W4 and increase in Y15. Copy number of other non-ColE1-type plasmids was not affected significantly in either mutant. It is likely that the copy number increase for most of the isolated mutants would also be specific for ColE1-type plasmids with the antisense regulation mechanism.

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