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FEMS Microbiology Letters 243 (2005) 227–233

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

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

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Received 31 August 2004; received in revised form 4 November 2004; accepted 9 December 2004

First published online 21 December 2004

Edited by W.J. Mitchell

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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0378-1097/$22.00
doi:10.1016/j.femsle.2004.12.015

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