Biochemical and Biophysical Research Communications 452 (2014) 32–35

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Screening of recombinant *Escherichia coli* using activation of green fluorescent protein as an indicator

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ARTICLE INFO

Article history:
Received 29 July 2014
Available online 17 August 2014

Keywords:
Cloning vector
Escherichia coli
Green fluorescent protein

ABSTRACT

A novel cloning vector that can be used to identify recombinant *Escherichia coli* colonies by activation of the green fluorescent protein gene (*GFP*) was constructed. Screening using the vector does not require special reagents. The recombinant plasmid activates *GFP*, and the rate of false-positive results is low.

1. Introduction

Blue–white screening is one of the most widely used methods for the selection of *Escherichia coli* (*E. coli*) harboring a recombinant plasmid [1,2]. The screen is based on insertional inactivation of the *lacZ* gene by a DNA fragment. It requires the inclusion of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) in agar medium. The β-galactosidase that is encoded by the normal *lacZ* gene produces a blue compound from the X-gal, whereas inactivated β-galactosidase does not. Therefore, nonrecombinant *E. coli* cells form blue colonies, whereas recombinant ones form white colonies. However, for clear discrimination between recombinant and nonrecombinant *E. coli* cells, high expression of β-galactosidase, which depends on the addition of isopropyl β-D-1-thiogalactoside (IPTG), is required. Furthermore, this screen has the defect of producing false-negative results for colonies in case of insertion of a small DNA fragment. To make the screening of recombinant *E. coli* cells more convenient, a cloning vector based on insertional inactivation of the green fluorescent protein (*GFP*) gene [3] has been developed [4–7]. Screening using the vector does not require special reagents because recombinant *E. coli* colonies do not fluoresce, whereas nonrecombinant ones fluoresce. However, the screen has the disadvantage of false-positive colony formation. Furthermore, finding a few nonfluorescent colonies among many fluorescent ones is somewhat troublesome. To solve these problems, a novel cloning vector was developed. The screen using this vector is based on the activation of *GFP*. The vector embodies a mechanism by which *GFP* is activated through the insertion of a DNA fragment harboring a stop codon. It was designated as an "off/on"-type vector. We propose that screening using the vector has the following advantages: (i) special reagents are not required to distinguish recombinant from nonrecombinant *E. coli*; (ii) selection is easier than that using the previous vectors because recombinant *E. coli* colonies exhibit fluorescence; and (iii) even if satellite colonies are formed, it is possible to select recombinants only. It is considered that recombinant *E. coli* with almost PCR products will fluoresce, because the probability of appearance of the stop codon in a PCR product is once in 64-bp. If the inserted DNA does not have a stop codon, it is necessary to introduce a stop codon in the fragment by PCR using primers harboring such a codon.

2. Materials and methods

2.1. pS147PGFP as a basic vector

The "off/on-type vector" was constructed by slight modification of pS147PGFP [8]. The map of pS147PGFP is shown in Fig. 1. pS147PGFP was constructed by insertion of the S147PGFP gene (*S147PGFP*) between *Eco*RI and *Hind*III restriction sites of a pkk223-3 expression vector (GE Healthcare, Buckinghamshire, UK; formerly Pharmacia). To enhance the fluorescence, the 147th residue, a serine, was substituted with proline (the methionine start codon is defined as the first residue).

2.2. Chemicals and instruments

Oligonucleotides for site-directed mutagenesis and PCR were synthesized by Life Technologies (Palo Alto, CA). Pfu turbo DNA

Abbreviations: E. coli, *Escherichia coli*; *GFP*, green fluorescent protein; IPTG, isopropyl-β-D-thiogalactopyranoside; LB, Luria–Bertani; PCR, polymerase chain reaction; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside.

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http://dx.doi.org/10.1016/j.bbrc.2014.08.038
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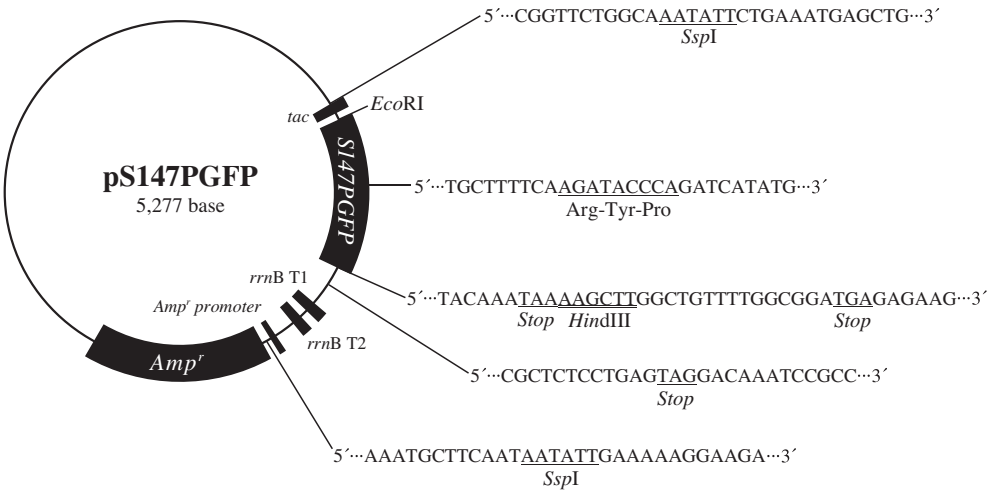

Fig. 1. Map of pS147PGFP. pS147PGFP was constructed by insertion of the S147PGFP gene between *Eco*RI and *Hin*dIII restriction sites in the pKK223-3 expression vector. The key sequences in this study are shown.

polymerase was purchased from Stratagene (La Jolla, CA, USA). *Dpn*I and the PureLink Quick Plasmid Miniprep Kit were purchased from Invitrogen (Carlsbad, CA, USA). *Ssp*I and the Mighty Cloning Reagent Set were purchased from Takara Shuzo (Shiga, Japan). PCR was performed with the iCycler (Bio Rad, Hercules, CA). Sequencing was performed with the ABI PRISM 3000 sequence analyzer (Applied Biosystems, Foster City, CA, USA). Fluorescence of colonies was evaluated with a 3UV transilluminator (UVP, Upland, CA, USA).

2.3. Construction of the "off/on"-type vector

A stop codon located at nucleotide positions 715–717 (5′-AAAT-AA-3′) of pS147PGFP was substituted with an *Ssp*I restriction site (5′-AATATT-3′) as a cloning site by site-directed mutagenesis [9,10]. The following primers were used for this substitution: forward primer, 5′-TGGATGAACTATACAAAAATATTAAGCTTGGCTGT TTTGGC-3′; and reverse primer, 5′-GCCAAAACAGCCAAGCTTAATA TTTTTGTATAGTTCATCCA-3′ (sequences for the substitution are underlined). The site-directed mutagenesis was achieved by cycling at the following temperatures: 16 cycles of 1 min at 95 °C, 30 s at 55 °C, and 13 min at 68 °C. Pfu turbo DNA polymerase was used for the reaction. After digestion of template plasmid by *Dpn*I at 37 °C for 1 h, *E. coli* JM109 cells prepared by a calcium chloride method [11] were transformed with the construct. The transformant was cultured on a Luria–Bertani (LB) agar plate containing ampicillin (final concentration of 50 μg/mL) at 37 °C overnight (approximately 20 h). After culture, the fluorescent colonies were picked and cultured in 10 mL of LB medium containing ampicillin (final concentration of 50 μg/mL). Their plasmids were purified using the PureLink Quick Plasmid Miniprep Kit. Sequencing the plasmids using the ABI PRISM 3000 sequence analyzer confirmed the correct introduction of the *Ssp*I restriction site. The next stop codon, TGA, at nucleotide positions 739–741, was substituted with TCA using the following primers: forward primer, 5′-GGCGGATC AGAGAAG-3′ and reverse primer, 5′-CTTCTCTGATCCGCC-3′ (sequences for the substitution are underlined) by site-directed mutagenesis. Unwanted *Ssp*I sites upstream and downstream of *S147PGFP* were substituted with other sequences by site-directed mutagenesis using the following primer sets: set 1, forward primer, 5′-GCTTCAATAACATTGAAAAAGG-3′; reverse primer, 5′-CCTTT TTCAATGTTATTGAAGC-3′ and set 2, forward primer, 5′-GGTTCT GGCAAATCTTCTGAAATG-3′; reverse primer, 5′-CATTTCAGAA GATTTGCCAGAACC-3′ (bases for the substitution are underlined). This plasmid was designed as the "off/on"-type vector (Fig. 2A).

2.4. Screening using the "off/on"-type vector

Approximately 200 ng of the vector was restricted using 1 unit of *Ssp*I at 37 °C for 1 h. Then, a 631-bp PCR product was ligated using the Mighty Cloning Reagent Set. After ligation, competent *E. coli* JM109 cells were transformed with the resultant plasmid. After overnight culture, some fluorescent colonies were observed under UV-A illumination (365 nm) using the 3UV transilluminator. Insertion of the DNA fragment was checked by PCR using the following universal primers: forward primer, 5′-GGATTACACATGGC ATGGATGAAC-3′ and reverse primer, 5′-CTCTCATCCGCCAAAAC AGCCAAG-3′.

3. Results and discussion

3.1. Construction of the "on/off"-type vector

First, factors that quench the fluorescence of S147PGFP were investigated because it was necessary for the "off/on"-type vector to have inactivated S147PGFP under the nonrecombinant condition. It was found in various studies (data not shown) that the exchange of a few stop codons downstream of *S147PGFP* inactivated its fluorescence. It was thus utilized as the "off/on"-type vector. *E. coli* JM109 cells transformed with the resulting plasmid in which the stop codon at nucleotide positions 715–717 was substituted with an *Ssp*I restriction site were not fluorescent. However, *E. coli* cells harboring the resulting plasmid in which the stop codon at nucleotide positions 739–741 was substituted with another codon were fluorescent. In other words, *S147PGFP* of the plasmid was inactivated (Fig. 2B). Generally, the fluorescence intensity of *GFP* tends to decrease depending on the hydrophobicity of the fused peptide [12]. These substitutions appended 70 amino acids to the C-terminus of S147PGFP, and 28 of these amino acids were hydrophobic. However, the average hydrophobicity of the peptide was approximately −0.64. It was unlikely to be sufficiently hydrophobic to decrease the fluorescence of S147PGFP. We speculated that the decrease in fluorescence depends on another factor. pS147PGFP originally had two unwanted *Ssp*I restriction sites upstream and downstream of *S147PGFP*. Substitution of these sites was required before practical use.

3.2. Screening using the "off/on"-type vector

After the construction, screening using the "off/on"-type vector was attempted. Some fluorescent colonies were observed among

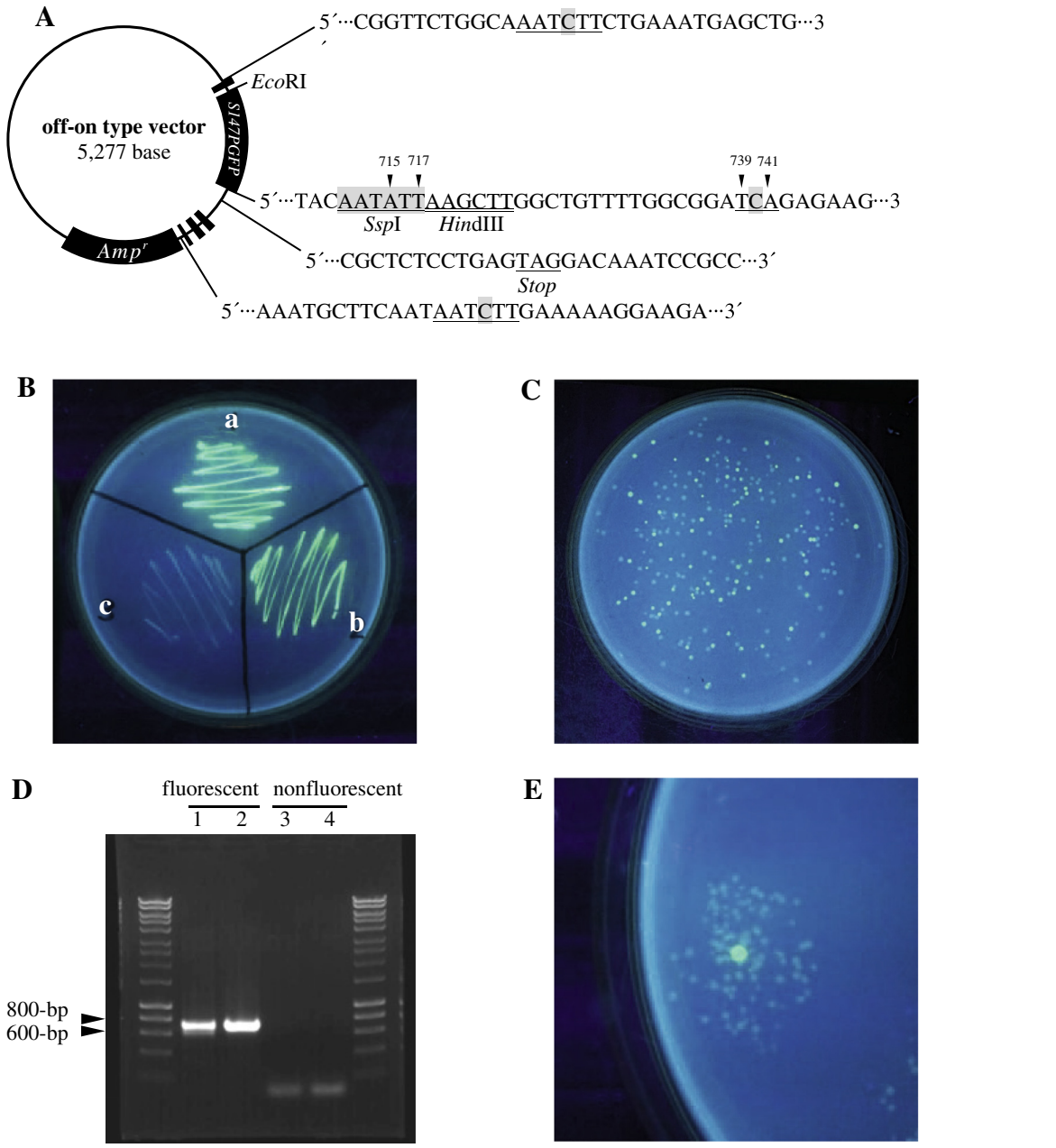

Fig. 2. Map of the "off/on"-type vector (A), fluorescence of *E. coli* cells harboring pS147PGFP and the "off/on"-type vector (B), screening of recombinant *E. coli* cells (C), amplification of the insert (D), and differentiation of recombinant *E. coli* from satellite colonies (E). The "off/on"-type vector was constructed by the introduction of an *Ssp*I restriction site at the 3′-terminus of *S147PGFP* and the inactivation of two stop codons downstream of *S147PGFP*. In addition, two incidental *Ssp*I restriction sites were inactivated. The substituted bases are shaded (A). The *E. coli* cells harboring pS147PGFP (B-a). The *E. coli* cells harboring pS147PGFP in which a stop codon at nucleotide position 715 (B-b). The *E. coli* cells harboring the pS147PGFP in which two stop codons at nucleotide positions 715–717 and 739–741 were substituted, namely the "off/on"-type vector (B-c). The colonies of recombinant *E. coli* cells were checked with the UV-transilluminator. Fluorescent colonies were candidates (C). Electrophoresis of the PCR products of insert is shown. Lanes 1, 2 and 3, 4 are PCR products that were amplified using plasmids extracted from fluorescent and nonfluorescent colonies, respectively (D). The recombinant colony was distinguishable from satellite colonies (E).

the nonfluorescent ones (Fig. 2C). Two fluorescent colonies were picked randomly and checked for insertion by amplification of a 631-bp DNA fragment by PCR. It was confirmed that the fluorescent colonies harbored recombinant plasmids (Fig. 2D). We propose that screening using the "off/on"-type vector has the following advantages: (i) special reagents are not required to distinguish recombinant from nonrecombinant *E. coli*; (ii) selection is easier than that using the previous vectors because recombinant *E. coli* colonies exhibit fluorescence; and (iii) even if satellite colonies are formed, it is possible to select recombinants only (Fig. 2E). In this experiment, the insert incidentally contained stop codons. However, it is considered that recombinant *E. coli* with other PCR products will fluoresce. The PCR product is not always ligated in the forward direction because *Ssp*I leaves blunt ends at the cloning site. Even though the PCR product does not have a stop codon in its forward sequence, it may have a stop codon in its reverse sequence. The probability of appearance of the stop codon in a PCR product is once in 64-bp. If the inserted DNA does not have a stop codon on either the forward or reverse direction, it is necessary to introduce a stop codon in the fragment by PCR using primers harboring such a codon.

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