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Green Fluorescent Protein as a Quantitative Reporter of Relative Promoter Activity in *E. coli*

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ABSTRACT

Green fluorescent protein (GFP) has become a valuable tool for the detection of gene expression in prokaryotes and eukaryotes. To evaluate its potential for quantitation of relative promoter activity in E. coli, *we have compared GFP with the commonly used reporter gene* lac*Z, encoding b-galactosidase. We cloned a series of previously characterized synthetic* E. coli *promoters into GFP and b-galactosidase reporter vectors. Qualitative and quantitative assessments of these constructs show that (a) both reporters display similar sensitivities in cells grown on solid or liquid media and (b) GFP is especially well suited for quantitation of promoter activity in cells grown on agar. Thus, GFP provides a simple, rapid and sensitive tool for measuring relative promoter activity in intact* E. coli *cells.*

INTRODUCTION

Green fluorescent protein (GFP) has enjoyed widespread use as a qualitative reporter of in vivo gene expression in prokaryotes and eukaryotes (9,13). Surprisingly few studies have reported the use of GFP as a quantitative reporter of promoter activity in prokaryotes (5,7,11, 14). In contrast, β-galactosidase (β-gal) has been used extensively as a reporter for quantifying gene expression in *Escherichia coli*. The ease of use of GFP makes it an attractive candidate as a reporter of promoter strength in *E. coli*.

Numerous variants of GFP are available (13). We chose to work with GFPuv because it offers several advantages over wild-type GFP: it is more soluble at high expression levels, less toxic and yields more intense fluorescence (3). (From this point, we will refer to GFPuv simply as GFP.)

To test the utility of GFP as a quantitative reporter, we constructed a promoterless GFP vector into which we inserted a series of previously studied synthetic promoters having varying activities (Table 1). Because β-gal has been so well studied as a quantitative reporter, we also tested the same promoters in a promoterless β-gal vector (6) to directly compare β-gal and GFP as reporters of promoter activity in *E. coli*. Here, we present the use of GFP as a rapid, simple and quantitative reporter of relative promoter activity in intact *E. coli* cells.

MATERIALS AND METHODS

Materials

We obtained DNA polymerase I (Klenow fragment), T4 DNA ligase, T4 polynucleotide kinase, *Taq* DNA polymerase, competent JM109 and a Wizard® Midi Prep Kit from Promega (Madison, WI, USA), restriction endonucleases, λ *Bst*EII marker and calf intestinal alkaline phosphatase from New England Biolabs (Beverly, MA, USA) and oligonucleotides from Life Technologies (Gaithersburg, MD, USA).

Cell Growth Conditions

For the plasmid copy number assay and for the GFP and β-gal liquid culture assays, cells were grown at 37°C in M9 minimal medium (8) supplemented with 1% glucose, 0.2% casamino acids, 1 μg/mL thiamine and 100 μg/mL ampicillin. (From this point, we will refer to this supplemented medium as M9 Glu+Caa.) LB broth was unsuitable for GFP liquid culture assays because of its high and variable background fluorescence (data not shown). For GFP plate assays, single colonies were streaked on LB agar plates supplemented with 100 μg/mL ampicillin, and plates were incubated at 37°C for 20 h followed by storage at 4°C.

Vectors

We obtained the pGFPuv plasmid from Clontech Laboratories (Palo Alto, CA, USA) and excised the *lac* promoter using *Sap*I and *Hin*dIII followed by

filling in the overhangs with DNA polymerase I (Klenow fragment) and religation. This promoterless vector was named pJC1.

To limit transcriptional readthrough, the bacteriophage T7 early transcriptional terminator (T7Te) (10) was directionally cloned downstream of the GFP coding sequence. The following complementary oligonucleotides were annealed and cloned between the *Eco*RI and *Spe*I sites in pJC1: *Eco*GFP, 5'-AATTCAAGCTTAAATGTAATCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGA-3′ and *Spe*GFP, 5′-CTAGTCGCAGAAAGGCCCACCCGAAGGTGAGCCAGTGTGATTACATTTAAGCTTG-3′. The resulting plasmid, which contains a promoterless GFP coding sequence with a T7Te terminator, was designated pJC2.

The pQF50 β-gal reporter vector (6) was used without modification.

Table 1 Promoters Used in This Study

Promoter[a]	-35 region	-10 region
4/6,4/6	TAGATATTTATCCCTTGCGGCGATAGATT	
5/6,4/6	TAGACA	TAGATT
5/6,5/6	TAGACA	TAGAAT
6/6,5/6	TTGACA	TAGAAT
6/6,6/6	TTGACA	TATAAT

[a]Promoter names reflect the number of bp in the -35 and -10 regions that match the *E. coli* consensus sequences for each region. For example, the 6/6,5/6 promoter matches the consensus in 6/6 bp in the -35 region and 5/6 bp in the -10 region. The sequence between the -35 and -10 regions, shown for 4/6,4/6, is the same in all constructs except for a single bp substitution in the 6/6,6/6 GFP construct (see Results).

Promoter Cloning

The synthetic promoters used in this study (Table 1) had previously been cloned into a different reporter vector (12). The promoters were amplified from this vector by PCR using primers that contained restriction sites for cloning the amplified promoters between the *Sph*I and *Kpn*I sites in pJC2 and pQF50. The pQF50 transformants were identified by the production of blue pigment on medium spread with 50 µL of X-gal (20 mg/mL in dimethylformamide) and/or by restriction analysis. The pJC2 transformants were identified by green fluorescence under longwave UV illumination and/or by restriction analysis. Plasmid DNA for each promoter clone was isolated using the Wizard Midi Prep Kit, and the sequence of each promoter region was determined by automated DNA sequence analysis.

Estimate of Relative Amounts of Plasmid in Each Strain

Cells were grown overnight in M9 Glu+Caa, diluted 25-fold in fresh M9 Glu+Caa and grown at 37°C to A_{600} = 0.8±0.05. Triplicate 1.5 mL aliquots were removed from each culture, plasmid DNA was isolated by alkaline lysis miniprep and an aliquot of each prep was run on a 1% agarose gel containing 0.5 µg/mL ethidium bromide. Each gel also included a lane with 250 ng of λ *Bst*EII molecular weight standard for estimation of plasmid mass. Gels were analyzed with 1D Gel Analysis software (Eastman Kodak, Rochester, NY, USA). Multiple plasmid isoforms were present

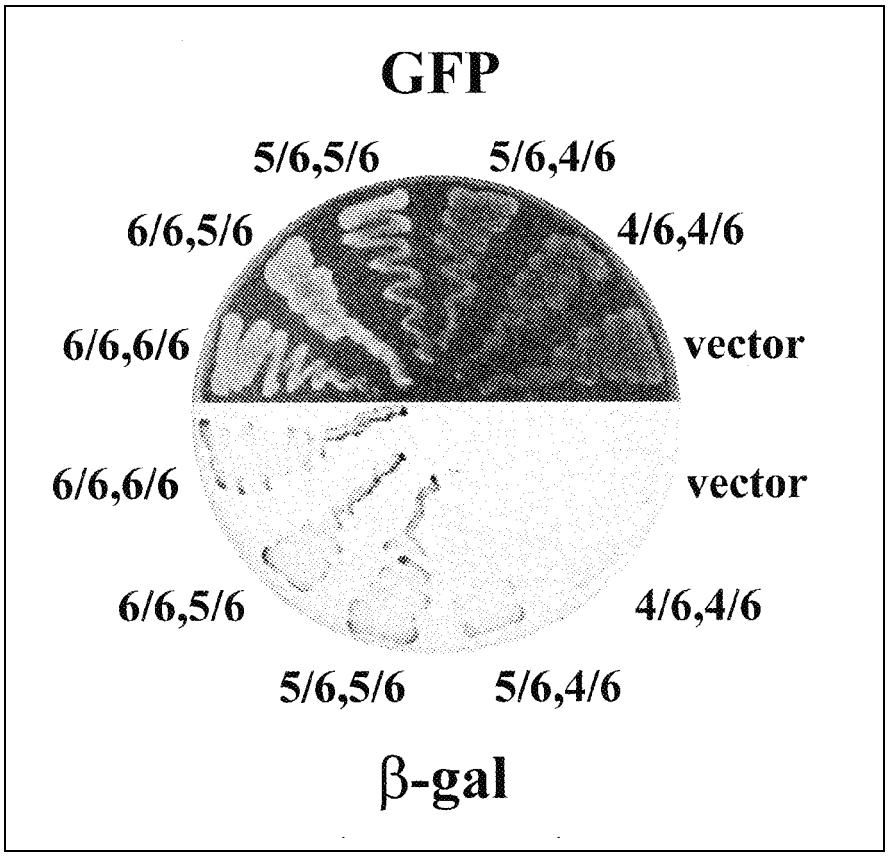

Figure 1. Expression of GFP and β-gal under the control of synthetic promoters. Strains containing plasmids that express GFP and β-gal driven by the indicated promoters were streaked on LB-ampicillin plates that contained X-gal and were grown at 37°C. Vector strains are promoterless. The upper portion of the figure shows GFP fluorescence produced by longwave UV illumination. The lower portion of the figure shows β-gal expression detected by the blue pigment produced by hydrolysis of X-gal.

in each lane, so the sum of the mass of DNA present in each lane was determined. The average plasmid DNA mass produced by each strain was then calculated and used to estimate the relative amounts of plasmid DNA in each strain.

Assay Conditions

β-gal assays were carried out essentially as described using chloroform and SDS to permeabilize the cells (1,8). Briefly, overnight cultures of cells grown in M9 Glu+Caa were diluted 25-fold into fresh M9 Glu+Caa and were grown at 37°C to A_{600} = 0.7 ± 0.1. Cells were harvested and the assay was conducted on 0.1, 0.2 and 0.3 mL of each culture using the pQF50 strain as the control. The optical density of the *o*-nitrophenol produced was measured at 420 nm in a Spectronic® 21D spectrophotometer (Milton Roy, Rochester, NY, USA) and was used to calculate units of β-gal (A_{420}/[min mL A_{600}]).

To assay GFP in cells grown on LB-ampicillin plates (see above for the cell growth conditions), plates were stored at 4°C for 24–28 h after growth at 37°C because this treatment produced a significant increase in fluorescence intensity (data not shown). Two plate-assay methods, (A) and (B), were used, each using pJC2 to determine background fluorescence.

(A) Plate scrape assay: Colonies were scraped from plates with an inoculating loop and resuspended in M9 Glu+Caa that lacked thiamine and contained chloramphenicol (CAM) (to prevent cell growth) to A_{600} = 0.20 ± 0.01. Approximately 20–40 colonies were used for each measurement. Duplicate fluorescence measurements of each sample were made at room temperature with an LS-3B Fluorescence Spectrometer (PE Biosystems, Foster City, CA, USA) with excitation at 395 nm and emission at 509 nm using the promoterless pJC2 strain as the blank. The response of the fluorometer was linear over approximately 3 orders of magnitude (data not shown). Parallel experiments were also conducted in which cells were scraped from the densest part of the streak.

(B) Charged-coupled device (CCD) camera assay: Images of single plates were obtained with a GDS-8000 cooled CCD camera system (UVP, Upland, CA, USA) using 365 nm epi-illumination and a green filter. Exposure time was 1/16 s, and the aperture was opened to maximum. Individual colonies were identified, and the fluorescence intensity was determined using the colony counting function in the GDS-8000 LabWorks® software.

To assay GFP in liquid culture, overnight cultures of cells grown in M9 Glu+Caa were diluted 50-fold into fresh M9 Glu+Caa and were shaken at 37°C until they reached the target OD (A_{600} = 0.50 ± 0.03). At this point, CAM was added to a final concentration of 200 µg/mL to stop protein synthesis and to allow complete folding of GFP, which folds slowly at 37°C (13). All strains reached the target absorbance within 4–5 h except for 5/6,5/6 that took 6.0–6.5 h. The cultures were started at staggered times so that all cultures reached the target OD within the same 60–90 min period. Increases in cell density and GFP fluorescence ceased within 1 h of the addition of CAM (data not shown). Cultures were kept shaking at 37°C for 1 h after CAM addition. The final A_{600} was determined (range 0.52–0.65), and cultures were diluted with M9 Glu+Caa containing CAM to A_{600} = 0.50 ± 0.02. GFP fluorescence was determined in a fluorimeter as described above. GFP fluorescence was stable for at least 2 h at room temperature.

RESULTS

The GFP reporter vector we developed, pJC2, encodes GFPuv, which is easily detected by longwave UV illumination (3). pJC2 was derived from the pGFPuv vector so it has several unique restriction sites (*Sph*I, *Pst*I, *Xba*I, *Xma*I, *Sma*I, *Kpn*I, *Asp*718I and *Age*I) to facil-

itate promoter cloning. We chose pQF50 as the β-gal reporter vector, which also has an extensive multiple cloning site (6).

We selected a previously characterized set of synthetic constitutive promoters that vary by a factor of >100 in terms of promoter strength (Table 1) and cloned each promoter into both vectors. DNA sequence analysis of these clones confirmed the expected -35 and -10 sequences. In addition, the 6/6,6/6 promoter cloned into pJC2 had acquired a T to A substitution at position -25, which would not be expected to affect promoter strength (2). Both pJC2 and pQF50 clones carrying the 4/6,4/6 promoter had unusual substitutions in which the CG dinucleotide at the -3 and -2 positions had been replaced by a single A. Wild-type 4/6,4/6 promoters have very low activity (12), so we did not use the 4/6,4/6 constructs in our quantitative assays.

Because plasmid copy number can be sensitive to the strength of a cloned promoter, we estimated the relative amounts of plasmid produced by each strain. The difference between high and low values for plasmid copy number for promoter clones in pJC2 was approximately 20%, while for pQF50 clones, the difference was approximately 35%. Because these differences were small, we did not consider them in evaluating the results of the assays.

All promoters except for 4/6,4/6 consistently produced visible levels of expression in strains streaked on agar plates (Figure 1) and in individual colonies (data not shown). It was possible to visually distinguish among the GFP strains, in terms of fluorescence, as follows: vector = 4/6,4/6 < 5/6,4/6 < 5/6,5/6 < 6/6,5/6 = 6/6,6/6. In contrast, for β-gal strains, the relative intensities of blue color were as follows: vector = 4/6,4/6 < 5/6,4/6 < 5/6,5/6 = 6/6,5/6 = 6/6,6/6. Therefore, on plates containing X-gal, GFP displayed a greater dynamic range than β-gal.

Figure 2 shows the results of quantitative analysis of relative promoter activity using GFP and β-gal reporter genes. GFP plate and β-gal liquid culture assays were able to distinguish among weak [5/6,4/6], moderate [5/6, 5/6] and strong [6/6,5/6 and 6/6,6/6] promoters. Therefore, GFP and β-gal are comparable in terms of sensitivity and dynamic range as quantitative reporters of relative promoter strength. Both methods used to quantify GFP expression on plates (i.e., plate scrape and CCD camera) are rapid, simple and in excellent agreement. In the plate scrape assay, essentially identical relative fluorescence values were obtained using either colonies or cells scraped from the densest part of the streak (data not shown). Interestingly, the absolute value of fluorescence in cells scraped from the densest part of the streak was 3–4-fold higher than the signal from colonies (data not shown).

Results obtained using a GFP liquid culture assay were inconsistent with results from GFP plate and β-gal liquid culture assays (Figure 2). Specifically, activity of the 5/6,4/6 promoter was undetectable in liquid culture, while the activity of the 5/6,5/6 promoter exceeded that of 6/6,5/6. In addition, we encountered difficulties with the growth of GFP-expressing strains in liquid culture including extremely poor growth of the 6/6,6/6 strain and variable growth rates (all strains reached the target absorbance within 4–5 h except for 5/6,5/6, which took 6–6.5 h). Curiously, all GFP strains seemed to grow well on LB-ampicillin plates at 37°C and all β-gal expressing strains grew well in liquid and on solid media.

DISCUSSION

GFP displays a sensitivity and dynamic range equivalent to β-gal as a reporter of relative promoter activity in both qualitative (Figure 1) and quantitative assays (Figure 2). This result is somewhat surprising because the lack of signal amplification inherent in GFP

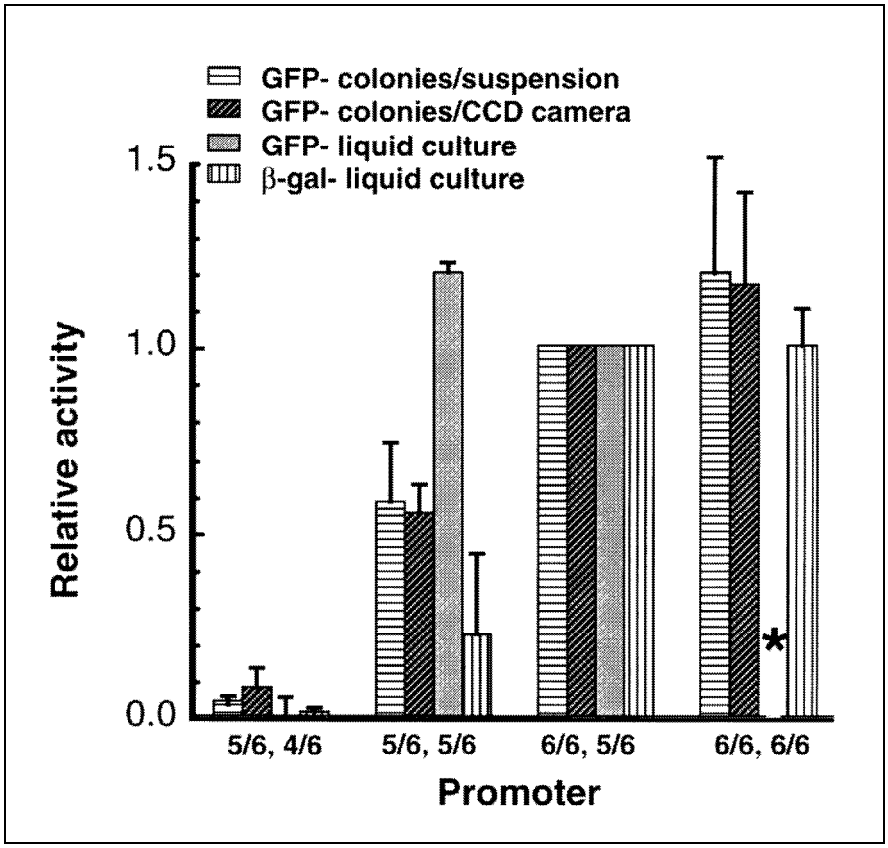

Figure 2. Quantitative analysis of relative promoter activity using GFP and β-gal reporters. GFP fluorescence and β-gal activity were quantified as described in Materials and Methods. For each assay, the data were obtained from three independent experiments and normalized to the activity of the 6/6, 5/6 promoter. For GFP-colonies/suspension and GFP-colonies/CCD camera, n=6 for each promoter; for GFP liquid culture, n=8 for 5/6, 4/6, n=15 for 5/6,5/6, and n=8 for 6/6, 5/6; and for β-gal, n=3 for each promoter. Error bars indicate standard deviation. *Not determined.

assays has been thought to be a shortcoming in comparison with enzymatic assays (9,13).

The two different GFP plate assays that we developed, plate scrape and CCD camera, are rapid, simple and sensitive. They allow ready quantitation of relative promoter strength over an activity range comparable to that for β-gal. In fact, GFP offers advantages over β-gal for assessing promoter activity. On plates, detection of GFP does not require the use of special media (e.g., media that contain X-gal). While a variety of sophisticated colorimetric plate assays for β-gal have been developed (8), all require the inclusion of one or more reagents in the growth medium. In addition, our GFP plate assays are much more rapid than the β-gal liquid culture assay because reporter activity is detected directly on plates and does not require a period of growth in liquid to a particular cell density. Weak and moderate strength promoters take no more time to assay than strong promoters in the GFP plate assays because the amount of reporter produced is measured directly. In contrast, weak and moderate strength promoters take longer to assay with β-gal because of the longer time required to produce measurable amounts of the product of β-gal activity, *o*-nitrophenol.

The GFP reporter vector that we have developed has a number of possible uses in addition to quantifying the activity of known promoters. For example, it should be useful as a vector for shotgun cloning and detection of promoters from a variety of eubacteria. In particular, the dynamic response of this vector in cells grown on agar should allow screening for promoters with a wide range of activities. The CCD camera assay should be ideal for such screens in which digitized images of colonies on plates could be subjected to image analysis to allow automated identification of colonies that express various levels of GFP. Similarly, the CCD camera assay should allow genetic screens for altered GFP expression levels to identify mutations affecting the regulation of a particular promoter.

Based on the data presented here, GFP may not be as good a reporter of promoter activity in a liquid culture assay, at least for constitutive promoters. We observed variable and sometimes poor growth of strains that express GFP in liquid culture, an effect not seen when these strains were grown on agar. The shortcomings of the liquid culture assay may stem from the apparent toxicity of GFP caused by the postulated release of H_2O_2 during chromophore formation (4). Interestingly, others have reported the apparently successful use of GFP as a quantitative reporter in liquid culture using inducible promoters (11,14). It would be interesting to see if engineering cells to express high levels of catalase (13) would reduce the postulated toxicity. Alternatively, GFP variants that are claimed to be non-toxic (e.g., blue fluorescent protein) may be better reporters for cells grown in liquid culture.

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