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Chapter 5

lacZ Reporter System as a Tool to Study Virulence Gene Regulation in Bacterial Pathogens

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Abstract

β-galactosidase assay has been established as one of the most widely used reporters and can be effectually exploited to study promoter activity of *Salmonella* and other pathogens under various conditions. This method includes a preliminary stage of fusing the target promoter to a promoter-less *lacZ* gene encoding for the enzyme β-galactosidase. Supplementation of the synthetic ONPG substrate results in the accumulation of a chromogenic product proportionally to the activity of the fused promoter. Here we demonstrate the usage of this reporter system to study the regulation of the *Salmonella* Type three secretion system effector gene *sseL* in *S.* Typhimurium [1].

Key words Beta-galactosidase (β-gal), *lacZ*, Lactose operon, *O*-nitrophenyl-β-D-galactoside (ONPG), Reporter, Gene regulation

1 Introduction

The *lacZ* gene from *Escherichia coli* encodes the enzyme β-galactosidase that cleaves the carbohydrate lactose to form glucose and galactose. Nevertheless, β-galactosidase can also recognize and hydrolyze a variety of chromogenic and fluorogenic synthetic substrates. One example is the colorless substrate *o*-nitrophenyl-β-D-galactoside (ONPG) that is hydrolyzed by β-galactosidase to yield galactose and o-nitrophenol. The byproduct o-nitrophenol has a yellow color, allowing quantitative spectrophotometric measurement. Since the production of o-nitrophenol is proportional to the concentration of β-galactosidase, the production of the yellow color can be used to determine indirectly the levels *lacZ* expression. This chemical reaction has been long exploited to create a reporter system that can monitor gene expression in an assay developed by Jeffrey Miller at 1972 [2]. This assay has been established as one of the most widely used reporters to study gene expression in molecular biology both in prokaryotes and eukaryotes. Basically, when a promoter-less *lacZ* gene is joined to a target promoter, the activity

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of the β-galactosidase can be used as a readout for the promoter activity. In this assay the synthetic substrate ONPG is added to the bacterial cells harboring the *lacZ* under the control of a promoter of interest that can be examined under various growth conditions (medium composition, environmental signals, stresses, etc.) and genetic backgrounds (e.g., in the absence of regulators). The amount of o-nitrophenol produced is dependent on the reaction time, and the number of cells generating a specific enzyme activity value expressed in Miller units [2].

Although this assay provides indirect measurements of promoter activity (as oppose to real-time qRT-PCR, for example), it is highly reproducible, straightforward, time-saving and does not require any expensive reagents and equipment, which may not be available to all labs. The *lacZ* fusion with the promoter of interest can be cloned into a low-copy number vector such as pMC1403 [3] or replace the chromosomal locus of the target gene. Here we demonstrate this reporter system to study the regulation of the *Salmonella* pathogenicity island (SPI) 2 effector gene *sseL* in *S.* Typhimurium and its regulation by the transcriptional regulator PhoP [1].

2 Materials

1. Luria–Bertani (LB) broth (Lennox) For 1 L: 10 g tryptone, 5 g yeast extract, 10 g NaCl (pH 7.0).
2. Low phosphate low magnesium minimal (LPM) medium: 80 mM MES (pH 5.8), 5 mM KCl, 7.5 mM $(NH_4)SO_4$, 0.5 mM K_2SO_4, 337 μM K_2HPO_4/KH_2PO_4 (pH 7.4), 20 mM $MgCl_2$, 38 mM glycerol, and 0.1% Casamino acids.
3. Bacterial genomic DNA purification kit.
4. Plasmid pMC1403 [3] (illustrated in Fig. 1) and plasmid extraction kit.
5. Hot start high fidelity DNA polymerase.
6. Primers to amplify the target promoter.
7. DNA gel extraction kit.
8. Restriction enzymes.
9. T4 DNA ligase.
10. X-Gal (5-bromo-4-chloro-3-indolyl-D-galactoside).
11. Sequencing primers: lacZ forward (5′-CCC CGA AAA GTG CCA CCT G-3′) and lacZ reverse (5′-GGA AGG GCG ATC GGT GCG GG-3′).

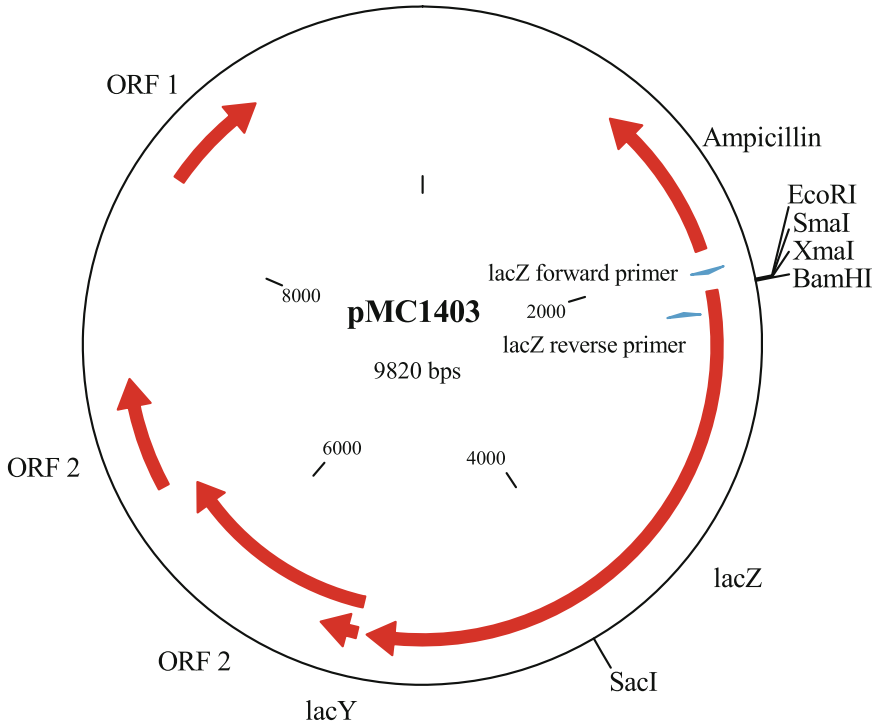

Fig. 1 Map of the *lacZ* reporter system pMC1403. A multiple cloning site upstream to the promoter-less *lacZ* contains the EcoRI, SmaI, XmaI, and BamHI sites. The position of *lacZ* forward and reverse primers is indicated as blue arrowheads

12. Ampicillin.
13. Z-buffer (for 1 L): 16.1 g of $Na_2HPO_4{\cdot}7H_2O$ (0.06 M), 5.5 g of $NaH_2PO_4{\cdot}H_2O$ (0.04 M), 0.75 g of KCl (0.01 M), 0.246 g of $MgSO_4{\cdot}7H_2O$ (0.001 M) and 2.7 mL β-mercaptoethanol (0.05 M). Prepare without β-mercaptoethanol, adjust pH to 7.0 and filter-sterilize (*see* **Note 1**).
14. P-buffer (for 200 mL): 2.136 g of $Na_2HPO_4{\cdot}2H_2O$ (0.06 M) and 1.248 g of $NaH_2PO_4{\cdot}2H_2O$ (0.04 M). Adjust to pH 7.0 and autoclave. Add 4 mg/mL ONPG before use for the amount of P-buffer needed.
15. *O*-nitrophenyl-β-D-galactoside (ONPG), 4 mg/mL in 0.1 M phosphate buffer, pH 7.0.
16. Toluene.
17. Stop solution: 1 M Na_2CO_3 (5.3 g in 50 mL).
18. Count up timer.

3 Methods

3.1 Construction of lacZ::Reporter Gene Fusion

1. Purify the bacterial genome of interest that will be used as the template for the PCR by bacterial genomic DNA purification kit or any other standard method.
2. Isolate the plasmid pMC1403 [3] using a plasmid mini extraction kit.
3. Amplify by PCR the target regulatory region using primers containing appropriate restriction sites (*see* **Note 2**).
4. Purify the PCR product using a gel extraction kit.
5. Cut plasmid pMC1403 and the PCR product with the same two restriction enzymes (we normally use EcoRI and BamHI; *see* Fig. 1) and purify the digested plasmid and the insert using DNA gel extraction kit and ethanol precipitation, respectively.
6. Ligate the insert into the plasmid using T4 DNA ligase at 16 °C overnight.
7. Transform the ligated construct into competent *Escherichia coli* cells (*see* **Note 3**) and plate the transformed bacteria onto LB plates supplemented with 100 μg/mL ampicillin and X-Gal (5-bromo-4-chloro-3-indolyl-D-galactoside), if the target promoter is active in-vitro you can expect to see the transformants as blue colonies.
8. Verify the cloning by restriction analysis and sequencing using the sequencing primers "*lacZ* forward" and "*lacZ* reverse."
9. Transform the plasmid into the appropriate *Salmonella* background strain.

3.2 Culture Growth and β-Galactosidase Assay

1. Grow *Salmonella* overnight with aeration at 37 °C.
2. The next day subculture by diluting the overnight culture 1:100 in fresh medium. Grow with aeration at 37 °C until the culture reaches 2–5 × 10^8 cells/mL (OD_{600} of 0.28–0.70), unless a different growth stage is wanted for the gene expression studies.
3. Place the culture on ice for 2 min to stop culture growth.
4. Measure and record the OD_{600}.
5. Into 2 mL test tubes prefilled with 0.9 mL of Z-buffer add 0.1 mL of the culture to a final volume of 1 mL. For low-activity promoters, one may add up to 0.5 mL of the culture, while complementing the total volume of the reaction to 1 mL with Z-buffer.
6. Add one drop of toluene to each tube and immediately vortex for 10 s (*see* **Note 4**).
7. Open the tube's caps and incubate the reaction tubes in a water bath set to 37 °C in a fume hood. This step is required to evaporate all the toluene from the solution.

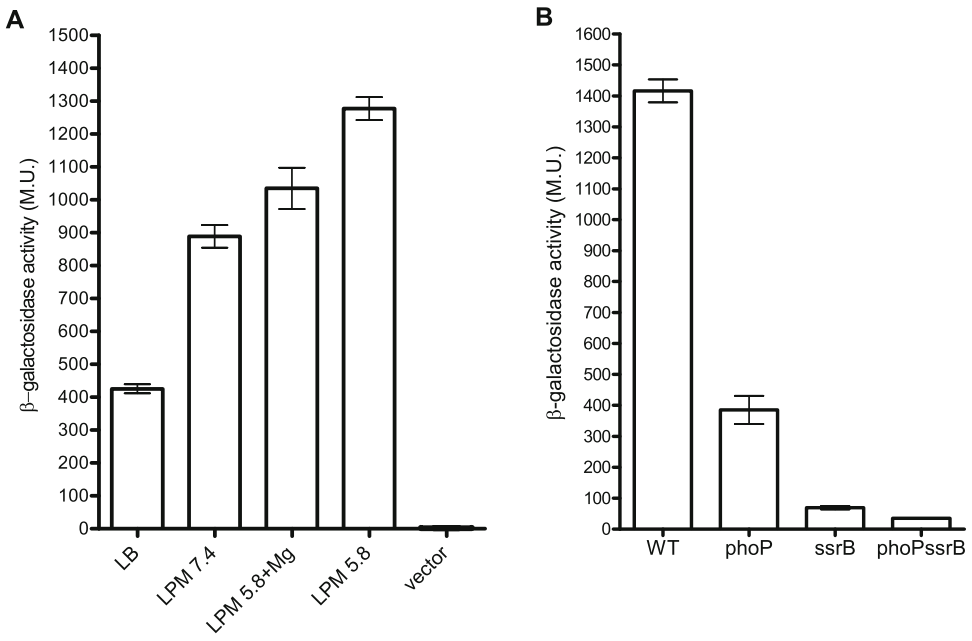

Fig. 2 *sseL::lacZ* is induced in response to low phosphate, low magnesium, and acidic pH environmental cues and is activated by PhoP and SsrB. (**a**) *S.* Typhimurium strains carrying *sseL:lacZ* were grown for 16 h at 37 °C in LB, LPM (pH 7.4) LPM (pH 5.8) supplemented with 10 mM $MgCl_2$, and LPM (pH 5.8), and were assayed for β-galactosidase activity presented in Miller units (M.U.). Basal *lacZ* expression of *S.* Typhimurium harboring pMC1403 (vector) that was grown in LPM (pH 5.8) is also shown. The induction of *sseL::lacZ* in LPM (pH 5.8) vs. LB can be appreciated. **(b)** β-galactosidase expression of *S.* Typhimurium wild-type (WT), *phoP*, *ssrB* and *phoP ssrB* double mutant strains harboring *sseL::lacZ* grown in LPM medium (pH 5.8). The positive regulatory role of PhoP and SsrB in *sseL::lacZ* expression is demonstrated. This figure is reproduced from Gal-Mor et al. (2011) [1] with permission from the publisher (PLOS Journals)

8. Equilibrate the tubes in a water bath at 28 °C for 5 min.
9. Add 0.2 mL of ONPG (4 mg/mL) to each tube and gently mix by inverting the tubes a few times. This is starting time of the reaction. Start running the timer.
10. Incubate the reaction at 28 °C until yellow color is clearly seen.
11. When a sufficient yellow color has been developed, stop the reaction by adding 0.5 mL of 1 M Na_2CO_3.
12. Record the stopping time of the reaction.
13. Centrifuge the tubes in a microfuge at 11,000 × *g* for 5 min to pellet cell debris (*see* **Note 5**).
14. Transfer 1 mL of the supernatant into cuvettes and measure the optical density at 420 nm for each reaction. The OD_{420} nm should be between 0.02–1.5 (*see* **Note 6**).
15. Calculate the β-galactosidase specific activity (Fig. 2) using the following formula:

$$\text{Miller units} = 1000 \times \frac{OD_{420}}{t \times v \times OD_{600}}$$

Where t = time of the reaction in minutes, v = volume of culture used in the assay (in mL; usually 0.1–0.5 mL).

4 Notes

1. Store the Z-buffer at 4 °C and add the required β-mercaptoethanol amount just before use to the appropriate volume of Z-buffer needed for the experiment.
2. We normally amplify a PCR fragment containing the entire intergenic region upstream to the target gene (or at least 500 bp upstream from the first methionine if the intergenic region is smaller than 500 bp) as well as the first seven amino acid codons of the target gene. If the target gene is organized in a polycistronic operon, and is not the first ORF in the operon, make sure that all the relevant regulatory sequence is cloned.
3. We recommend to use the *E. coli* K-12 strain MC1061, containing a null deletion of the entire *lac* operon.
4. This step is required to permeabilize the bacteria envelope allowing ONPG to penetrate into the cytoplasm where the β-galactosidase is expressed.
5. This step is important to minimize light scattering by cell debris that can affect the absorbance at OD_{420}.
6. The absorbance at OD_{420} should be between 0.02 and 1.5 to be within the linear range of the assay. If a reading is above 1.5, dilute the reaction with Z-buffer and multiply the newly measured OD_{420} by the dilution factor.

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