

Using pMJG2 and related vectors to create and use single-copy GUS promoter fusions in *Listeria monocytogenes*

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Attached & related documents:

maps of pMJG2 & pMJG3

writeup of results as of 6/3/04

Introduction

The pMJG2 vector (see attached map) allows the construction of reporter fusions between any promoter and the reporter gene β -glucuronidase (GUS) and the integration in single copy of those fusions into the *L. monocytogenes* chromosome. This vector is based on pPL2 (Lauer et al., 2002), which uses a phage integrase to integrate itself at a tRNA^{Arg} site. This document will describe the construction and use of pMJG2-based fusion vectors. Also included is a brief summary of results obtained to date using this system.

There are three stages to these experiments: cloning the promoter of interest into pMJG2 to build the fusion vector, construction of *L. monocytogenes* fusion strains, and measuring GUS activity. Protocols for each stage are included below.

A similar pPL2-based vector for construction of luciferase fusions also exists. It is called pMJG4, and is stored in G1-219. Most of the following protocols could also be used with pMJG4, with some modification. However, pMJG4 has not been tested as thoroughly as pMJG2.

A. Construction of fusion vectors

Required Materials

Strains (Tower 9, Box 8)

FSL G1-210 = *E. coli* SM10 conjugation donor

FSL G1-221 = *E. coli* TOP10 + pMJG2 (Cm^R)

Primers (Tower A, Box 7)

JUPR (AAT TGC CCG GCT TTC TTG TAA C)

2003.2.3A (TGC ATG TGT CAG AGG TTT TCA)

M13F & R: provided by sequencing facility

Kits

Qiagen Plasmid Miniprep Kit (Catalog # 27106)

Qiagen Gel Extraction Kit (Catalog # 28704)

Invitrogen TOPO TA Cloning Kit (Catalog # 45-0641)

Cloning

LB/Ap(50µg/ml) plates: ~7 per construct

LB/Ap(50µg/ml) broth

Restriction enzymes and appropriate buffers (probably *SalI* and *BamHI*)

Electroporation

ice cold sterile 10% glycerol: 200ml per preparation

LB/Cm(20µg/ml) plates: 2 per electroporation

SOC Medium: 1ml per electroporation

Recipe:

2.0g tryptone peptone

0.5g yeast extract

0.06g NaCl

0.02g KCl

90ml dH₂O

0.2 g MgCl₂

0.12g MgSO₄

0.36g glucose

* adjust pH to 7.0

* adjust volume to 100ml

* filter sterilize

Protocol

There is no specific protocol for construction of vectors, but the techniques used are mostly standard molecular biology methods. See Maniatis for additional protocols. What follows is an outline of how I went about construction of fusions.

1. Amplify promoter of interest with PCR. Design your primers to incorporate restriction sites at each end of your fragment which will allow you to insert it in the correct orientation in pMJG2. I used *SalI* as the upstream restriction site and *BamHI* as the downstream site with good success. Use Taq Gold to minimize the chance of PCR-introduced point mutations. You will only need about 5µl of PCR product, so a small PCR reaction is more than adequate.
2. Clone your PCR product with the Invitrogen TOPO TA Cloning kit, which allows you to rapidly clone an unprocessed PCR product into a high-copy ampicillin resistant plasmid. Transform the ligation mixture into Invitrogen TOP10 supercompetent *E. coli*. Follow the manufacturer's instructions.

3. Prepare enough of your intermediate vector (promoter in pCR2.1 TOPO) and of pMJG2 for the next step using the Qiagen Plasmid Miniprep kit. This is trivial with the intermediate vector, just follow normal procedure. However, pMJG2 is a low-copy plasmid, so you will need to grow up 10ml of overnight culture and prepare plasmid from all of it. I used 2 Qiagen columns and eluted both of them into the same final tube with 30µl each. (See manufacturer's instructions.)
4. Digest both plasmids with the appropriate restriction enzymes. (Probably *SalI* and *BamHI*, which can be used together in a double digest: see NEB Catalog.) Be sure to let the digest go long enough to ensure complete digestion.
5. Gel purify the digested vector and insert, using the Qiagen Gel Extraction kit.
6. Ligate your purified, digested vector and insert fragments with T4 DNA Ligase. (Protocol in Maniatis.)
7. Electroporate ligation mixture into *E. coli* SM10 (G1-210):

Preparation of electrocompetent cells

1. Inoculate 1ml of fresh overnight culture of *E. coli* into 100ml of LB broth in a sidearm flask.
2. Grow cells at 37°C with shaking to an OD₆₀₀ of 0.5 – 0.7 (approximately 3 hours).
3. Chill cells on ice for 20 minutes. Keep cells and containers as cold as possible for all subsequent steps.
4. Transfer cells to a cold sterile centrifuge bottle and spin at 4000 x g for 15 minutes at 4°C.
5. Discard supernatant and gently resuspend pellet in 100ml of cold sterile 10% glycerol.
6. Centrifuge at 4000 x g for 15 minutes at 4°C.
7. Discard supernatant and resuspend pellet in 50ml of cold sterile 10% glycerol.
8. Centrifuge at 4000 x g for 15 minutes at 4°C.
9. Discard supernatant and resuspend pellet in 4ml of cold sterile 10% glycerol.
10. Centrifuge at 4000 x g for 15 minutes at 4°C.
11. Discard supernatant and resuspend pellet in 200µl of cold sterile 10% glycerol.
12. Aliquot 40µl of cell suspension to chilled 1.5ml microfuge tubes and either use immediately or store at -80°C. Cells are reputedly stable for at least 6 months.

Electroporation

1. Thaw electrocompetent cells on ice.
2. Add 1 to 2µl of ligation mixture to 40µl of cells. Mix well and incubate 1 minute on ice.
3. From the Home screen on the Gene Pulser electroporator, open the Pre-set Protocols screen, then the Bacterial Protocols screen. Open the *E. coli*, 1mm cuvette Protocol Detail screen. (C = 25µF, PC = 200Ω, V = 1.8kV)
4. Transfer mixture of cells and DNA to a cold cuvette and tap suspension to the bottom.

5. Place cuvette in ShockPod and pulse once. Time constant should be close to 5 milliseconds.
(Lower works, much higher doesn't.)
6. Remove the cuvette from the chamber and immediately add 1ml room temperature SOC.
7. Quickly but gently resuspend cells. Delaying transfer causes a rapid decline in transformation efficiency.
8. Transfer cell suspension to a sterile microfuge tube and incubate at 37°C with shaking for 1 hour.
9. Plate 50µl, 100µl, and the rest of the suspension on LB/Cm(20) plates and incubate overnight at 37°C.

B. Construction of *Listeria* strains

Required Materials

Strains (Tower 9, Box 8, among other places)

E. coli SM10 containing pMJG2 with inserted promoter (from part A above)

L. monocytogenes 10403S

FSL A1-254 (*L. monocytogenes* 10403S $\Delta sigB$)

FSL B2-046 (*L. monocytogenes* 10403S $\Delta prfA$)

any other *L. monocytogenes* strain you intend to use as a background strain (must be streptomycin resistant: all 10403S derivatives are Sm^R)

Primers (Tower A, Box 7)

NC16 (GTC AAA ACA TAC GCT CTT ATC)

PL95 (ACA TAA TCA GTC CAA AGT AGA TGC)

2003.4.1A (GAA TAA GGG ACA GTG AAG AAG G)

JUPR (AAT TGC CCG GCT TTC TTG TAA C)

2003.2.3A (TGC ATG TGT CAG AGG TTT TCA)

Integration

sterile 47mm diameter 0.45µm HA-type filters (Millipore): 1 per integration

150ml Analytical Filter Unit (Nalgene, catalog # 130-4045): 1 per integration

BHI broth

BHI plates

BHI plates + 7.5µg/ml chloramphenicol & 200µg/ml streptomycin

LB top agar (0.7% Bacto-Agar)

LB/Cm(20µg/ml) broth

Protocol

1. Grow donor culture (*E. coli* SM10 containing pPL2-derived plasmid) and *L. monocytogenes* recipient strains overnight in 5ml broth. Donor culture is grown in LB/Cm(20) and recipient strains are grown in BHI.
2. Inoculate 5ml fresh broth with 100µl of each overnight culture. (You may need more than one tube of donor culture. See step 4 below.)
3. Grow cultures to OD₆₀₀ = 0.5 – 0.6 at 30°C with shaking (approximately 3.5 hours). Give *Listeria* strains about a 40 minute head start so that all strains reach desired OD₆₀₀ roughly simultaneously.
4. Mix 2.5ml of donor culture with 1.5ml of recipient culture.
5. Filter mixture onto sterile Millipore filter using analytical filter unit and vacuum pump.
6. Wash filter with 10ml BHI broth.
7. Place filter on a prewarmed (30°C) BHI plate and incubate for 2 hours at 30°C.
8. Resuspend cells gently in 2.5ml BHI broth.
9. Plate 50µl and 100µl aliquots in 3ml LB top agar (tempered to 46°C) on prewarmed (30°C) BHI/Cm(7.5), Sm(200) plates.
10. Incubate plates overnight at 30°C.
11. Transfer plates to 37°C and incubate overnight.
12. Pick large individual colonies and streak for isolation on BHI/Cm(7.5), Sm(200) plates.
13. Make lysates of colonies for screening (see standard lab protocol).
14. Screen for integration and correct insert with PCR:

PCR A:

primers: NC16 & PL95

conditions: 30 cycles:

1 min @ 94°C

1 min @ 55°C

1 min @ 72°C

expected results: 499bp band = vector has integrated

PCR B:

primers: PL95 & 2003.4.1A

conditions: 30 cycles:

1 min @ 94°C

1 min @ 55°C

1 min @ 72°C

expected results: 700bp band = multiple integration, no band = good

PCR C:

primers: JUPR & 2003.2.3A

conditions: 25 cycles:

1 min @ 94°C

1 min @ 58°C

1 min @ 72°C

expected results: amplifies promoter region, include positive control

15. You may sequence PCR products A & C to confirm your insert.

16. Freeze integrants at -80°C.

C. Assaying GUS activity

Required Materials

AB_{light} Buffer:

10.45g K₂HPO₄

5.44g KH₂PO₄

5.85g NaCl

1 L dH₂O

* adjust pH to 7.0

* autoclave 30 minutes at 121°C

AB/DMSO = 35ml AB_{light} + 100µl DMSO (dimethyl sulfoxide)

MUG Stock:

1mg/ml MUG (4-methylumbelliferyl-β-D-glucuronide, Sigma catalog # M9130) dissolved in
DMSO

* make at least 5 ml

* store at 4°C (note: DMSO freezes at this temperature)

BHI plates + 7.5µg/ml chloramphenicol & 200µg/ml streptomycin

BHI/Cm(7.5µg/ml) broth

Stimulation media (varies, see below)

Bead-beater tubes:

2ml screw cap tubes w/ o-ring (Quality Scientific Plastics, catalog #522)

0.5mm zirconia/silica beads (Biospec Products #11079105Z)

1mm zirconia/silica beads (Biospec Products #11079101Z)

* mix equal weights of each bead type

* aliquot 1.5g of mixture to tubes

* autoclave tubes to sterilize

Bio-rad Protein Assay Reagent (catalog #500-0006)

96-Well Plates:

black flat bottomed (Corning catalog #3915)

clear flat bottomed (Corning catalog #3596)

Protocol

1. Prepare 4-methylumbelliferone (MU) standard curve:

* Do not do this on the same day as you are planning to read your assay, since MU is highly fluorescent and may contaminate your results.

* MU is stored at room temperature above the PFGE machine.

- a. Dissolve 28mg MU in 1ml DMSO.
- b. Add 50 μ l of a. to 450 μ l of DMSO.
- c. Add 10 μ l of b. to 3.5ml AB_{light} = 40 μ M, 4800pmol in 120 μ l
- d. Add 1.5ml of c. to 3.25ml AB/DMSO = 12.6 μ M, 1500pmol in 120 μ l
- e. Add 1ml of c. to 3.75ml AB/DMSO = 8.4 μ M, 1000pmol in 120 μ l
- f. Add 1ml of c. to 8.5ml AB/DMSO = 4.2 μ M, 500pmol in 120 μ l
- g. Add 2ml of f. to 6ml AB/DMSO = 1.04 μ M, 125pmol in 120 μ l
- h. Add 2ml of g. to 1.3ml AB/DMSO = 0.63 μ M, 75pmol in 120 μ l
- i. Add 1ml of h. to 500 μ l AB/DMSO = 0.42 μ M, 50pmol in 120 μ l
- j. Add 500 μ l of i. to 2.125ml AB/DMSO = 0.08 μ M, 10pmol in 120 μ l

* Store solutions c. – j. at 4°C in the dark (wrapped in foil).

* Use 5 of the above solutions as your standard curve. I recommend 1000pmol, 500pmol, 125pmol, 75pmol, and 10pmol, but it may vary depending on the strength of your promoters.

2. Streak *L. monocytogenes* strains with integrated pMJG2-derived plasmids from frozen stock on BHI/Cm(7.5), Sm(200) plates. Incubate 1-2 days at 37°C.

3. Inoculate 5ml BHI/Cm(7.5) broth with a large colony of each strain of interest. Incubate overnight at 37°C with shaking.

4. Inoculate 50 μ l of overnight cultures into 5ml fresh BHI/Cm(7.5) broth. Incubate at 37°C with shaking and monitor OD₆₀₀.

5. Once OD₆₀₀ ~ 0.4 (approximately 6 hours), inoculate 25 μ l of cultures into 5ml of fresh stimulation media. This will depend on the goal of your experiment, but stimulation media have in the past included:

BHI/Cm(7.5) + 0.2% (w/v) activated charcoal powder

BHI/Cm(7.5) pretreated with activated charcoal, then filtered

BHI/Cm(7.5) + 25mM cellobiose

BHI/Cm(7.5) + 0.3M NaCl

6. Incubate 18hrs at 37°C with shaking. (Note: You may wish to vary this incubation time or collect cells at several time points, which will require you to adjust the total volumes in step 5.)

7. Harvest 1.1ml aliquots of cultures by centrifuging 5 min at 10,000 rpm.

8. Discard supernatant and resuspend in 1ml AB_{light}.
9. Centrifuge 5 min at 10,000 rpm.
10. Discard supernatant and resuspend in 1.1ml AB_{light}. Suspensions may be stored frozen at -80°C.
11. Transfer 1ml of suspension to bead-beater tube containing sterile glass beads (see Materials section). Make sure o-ring is in place.
12. Vortex 60 sec at 4,200 rpm in bead-beater. (Setting: T = 6, S = 42) Put tubes on ice.
13. Centrifuge 5 min at 10,000 rpm and transfer supernatant (approximately 700µl) to clean microfuge tubes. Store frozen at -20°C. This supernatant will be used for GUS activity measurements and protein quantifications.
14. Make MUG working stock:
 - 800µl 1mg/ml MUG in DMSO
 - 1200µl DMSO
 - * gives 0.4mg/ml MUG in DMSO
15. Turn on Fusion machine to warm up and select the program “Celine’s GUS assay” (excitation 365nm, emission 455nm, sensitivity = “Low Range”).
16. Transfer 100µl in duplicate to wells of a 96-well optiplate (black, flat-bottom) with 100µl of AB_{light} (in duplicate) as a blank.
17. Add 20µl of MUG working stock to each well. Record exact time of addition. Use a multipipettor. It will save a lot of trouble.
18. Incubate 40 minutes in the dark at room temperature. Cover the plate to prevent evaporation.
19. Add 120µl of standards in duplicate to plate.
20. Incubate 15 – 20 minutes in the dark at room temperature.
21. Read plate in Fusion machine (record time), and save RFU for each well. (The Fusion machine will output an Excel file. Ask someone about the software; it’s startlingly counterintuitive.)
22. Set Fusion machine to the “Ute’s Total Protein” program. (Absorbance at 600nm.)
23. In a clear, flat-bottom 96-well plate, prepare protein assay standard curve:
 - a. dilute NEB 10mg/ml BSA solution to 80µg/ml in AB_{light}. (1ml of a 1:100 dilution + 250µl AB_{light})
 - b. Add to wells in duplicate:

µg BSA/160µl	80µg/ml BSA stock	AB _{light}
1.28	16µl	144µl
2.56	32µl	128µl
5.12	64µl	96µl
10.24	128µl	32µl
12.8	160µl	0µl

24. Add 80µl of each GUS supernatant + 80µl of AB_{light} in duplicate to wells of plate. (1:2 dilution) If you are using an early time point or a medium which doesn't allow a lot of growth, you may not want to dilute your sample.
25. Add 40µl of Bio-rad protein assay reagent to each well. Mix thoroughly, but be careful not to introduce bubbles, which will really screw up your absorbance readings. Spinning down the plates helps get rid of bubbles.
26. Incubate plate at room temperature for about 5 minutes.
26. Save Excel file with your OD₆₀₀ data.
27. Use your standard curves to calculate RFU/120µl for GUS assays and µg protein/160µl for protein quantifications for each treatment.
28. Calculate GUS Units (pmol MU hydrolyzed / [log protein x ml x minutes]).
29. Repeat steps 3 – 28 and calculate average GUS Units for each construct.

Results to Date

The following constructs have been completed: (Tower 9, Boxes 8 & 12)

FSL Number	Promoter	Background Strain
G1-231	No promoter	10403S
G1-239	No promoter	10403SΔ <i>sigB</i>
G1-233	No promoter	10403SΔ <i>prfA</i>
G1-232	<i>Phly</i>	10403S
G1-240	<i>Phly</i>	10403SΔ <i>sigB</i>
G1-234	<i>Phly</i>	10403SΔ <i>prfA</i>
G1-235	<i>PrpoB</i>	10403S
G1-237	<i>PrpoB</i>	10403SΔ <i>sigB</i>
G1-236	<i>PrpoB</i>	10403SΔ <i>prfA</i>
G1-251	<i>PrsbV</i>	10403S
G1-250	<i>PrsbV</i>	10403SΔ <i>sigB</i>
G1-252	<i>PrsbV</i>	10403SΔ <i>prfA</i>
G1-259	<i>PprfA</i>	10403S
G1-260	<i>PprfA</i>	10403SΔ <i>sigB</i>
G1-261	<i>PprfA</i>	10403SΔ <i>prfA</i>
G1-262	<i>PprfA</i> ΔP1(-10)	10403S
G1-263	<i>PprfA</i> ΔP1(-10)	10403SΔ <i>sigB</i>
G1-264	<i>PprfA</i> ΔP1(-10)	10403SΔ <i>prfA</i>
G1-265	<i>PprfA</i> ΔP2(-10)	10403S
G1-266	<i>PprfA</i> ΔP2(-10)	10403SΔ <i>sigB</i>
G1-267	<i>PprfA</i> ΔP2(-10)	10403SΔ <i>prfA</i>
G1-268	<i>PprfA</i> ΔP1P2(-10)	10403S
G1-269	<i>PprfA</i> ΔP1P2(-10)	10403SΔ <i>sigB</i>
G1-270	<i>PprfA</i> ΔP1P2(-10)	10403SΔ <i>prfA</i>
G1-273	<i>PopuCA</i>	10403S
G1-274	<i>PopuCA</i>	10403SΔ <i>sigB</i>
G1-275	<i>PopuCA</i>	10403SΔ <i>prfA</i>

General Observations

- At 18 hours, most promoters produce enough GUS activity to be read easily by the Fusion machine. It may be advisable to concentrate cultures to measure activity at earlier timepoints or in less rich media.
- *PrpoB* is more or less constitutive under all conditions tested. However, activity is fairly low, in contrast to TaqMan results which suggest constitutively high *rpoB* expression.
- *PrsbV* and *PprfA_{P2}* do not appear to produce enough transcript to be observed using this system. No GUS activity was observed from either of these promoters. They were confirmed by sequencing, so the constructs are correct.
- BHI containing 0.2% (w/v) activated charcoal powder is more consistently effective at activating *Phly* expression than BHI pretreated with activated charcoal. Pretreated BHI, in my hands, produced very inconsistent results, although Nancy Freitag apparently swears by it. See Ermolaeva et al. 2004 for more info on charcoal.

Special Features

There are a few features of the pMJG2 system which are not immediately obvious but which may be useful for some projects. First is the simple note that these pPL2-based plasmids can be integrated into any *L. monocytogenes* strain. The protocol described above assumes that the *Listeria* strain will be streptomycin resistant (all 10403S derivatives are), but this is not strictly required. Plating the conjugation mixture on Oxford plates with chloramphenicol should be adequate to allow growth of conjugants and kill *E. coli* donor cells. Note that I haven't actually tried this.

The other potentially useful feature lies in the construction of pMJG3, the *PrpoB-gus* fusion vector (stored in *E. coli* SM10 as G1-230). Instead of inserting *PrpoB* into the *SalI* and *BamHI* sites of pMJG2 (as was done for all other promoters), *PrpoB* was inserted into the *SalI* and *KpnI* site upstream of the polylinker. This means that there is a complete polylinker between the *PrpoB* promoter and the *gus* gene. Since *PrpoB* is constitutively active, this should allow determination of both positive and negative transcriptional regulation for any promoter inserted into the polylinker. This was intended to test the mechanism of negative regulation at *PprfA_{P2}*, but I didn't have time to make those constructs. See attached map of pMJG3 for details.

Potential Future Experiments

There are a wide range of potential experiments that could be done using the system described above. Some possibilities are listed below:

1. The experiments that have been done (see attached) need to be repeated and cleaned up to make them publishable.

2. A timecourse experiment measuring *PprfA* (& mutants) expression at 4, 8, 12, and 18 hours. (4 & 8 hours straddle beginning of stationary phase for *Listeria*. Some fiddling with the protocol may be necessary to see adequate expression at early timepoints.
3. The *PopuCA* promoter needs to be tested as a σ^B control in BHI + NaCl & other media.
4. The *PprfA* promoters should be tested in BHI + cellobiose.
5. It would be interesting to examine gene expression in BHI + activated charcoal and NaCl or cellobiose. This might allow determination of the order of regulation by various environmental conditions.
6. The *PprfA*(-35) deletions could be put into pMJG2. The strains containing these deletions are stored in Céline's freezer box. (FSL N2-011, -012, and -013) This would hopefully allow dissection of different promoter elements, including the PrfA-box which overlaps the -35 region of the P2 promoter.
7. Various promoter fusions could be put into other regulatory mutants as reporters, including $\Delta ctsR$, $\Delta clpC$, $\Delta hrcA$, $\Delta lisR$, $\Delta cesR$, $\Delta rsbV$, and $\Delta rsbT$. Some of these might require construction of new control fusions, like a *PclpP-gus* fusion as a *ctsR* reporter.
8. To improve sensitivity of detection of *gus* transcripts, Ute has developed a set of *gus* TaqMan primers and probes. If detection of low levels of activity continues to be a problem, this might help.
9. A parallel system using *lux* instead of *gus* as a reporter gene exists. The vector pMJG4 is stored in *E. coli* TOP10 as G1-219. Barbara has done some work with this system and the *PopuCA* promoter.