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1 *In Springer Methods and Molecular Biology Protocols Book, Biology of Yersinia Infection*

2 **Title**

3 **Real-time monitoring of *Yersinia pestis* promoter activity by bioluminescence imaging**

4 Anne Derbise, Olivier Dussurget, Elisabeth Carniel and Javier Pizarro-Cerdá

5

6 Running title

7 Real-time monitoring of *Yersinia pestis* promoter activity

8

9 **Abstract**

10 Bioluminescence imaging has become a major strategy for real-time analysis of dynamic
11 biological processes. In particular, bioluminescent reporter microorganisms have been
12 designed to advance our understanding of infectious diseases. Non-invasive monitoring of
13 light-emitting pathogenic bacteria has revealed novel features of pathogenesis and enabled
14 quantitative and qualitative analysis of antibacterial therapies. Transcriptional gene fusions
15 using the bacterial luciferase operon *luxCDABE* as a reporter have been successfully used to
16 monitor gene expression *in vitro* and *in vivo*, leading to valuable applications and major
17 findings. In this chapter, we describe the construction of *Yersinia pestis* strains bearing a
18 chromosomal copy of the *luxCDABE* operon under the control of promoters regulated by
19 temperature and their application to quantify gene expression in real-time in bacteria growing
20 *in vitro* and in a murine bubonic plague model.

21

22 **Key words** Bioluminescence, real-time imaging, reporter system, promoter activity, plague,
23 *Yersinia pestis*, mice

24

25

26 **1. Introduction**

27 Bioluminescence is the production and emission of light by a living organism. With the
28 development of highly sensitive photon detectors, *in vivo* bioluminescence imaging (BLI)
29 techniques have been particularly useful for real-time noninvasive tracking of bacteria within
30 animal models.

31 Bioluminescent imaging has allowed monitoring of the proliferation and dissemination of
32 *Yersinia pestis* in real-time in different murine models, such as bubonic and pneumonic
33 plague [1-3]. In these studies, bioluminescence was produced by the *Photobacterium*
34 *luminescens* luciferase which is encoded by the genes *luxA* and *luxB* that catalyzes the
35 oxidation of a reduced flavin mononucleotide by oxygen leading to the production of photons
36 that can be quantified. The substrate is recycled by the enzymatic complex encoded by genes
37 *luxD*, *luxC* and *luxE*, allowing detection of bacteria expressing the *luxCDABE* operon without
38 addition of exogenous substrate. The *P. luminescens luxCDABE* operon provides several
39 advantages when expressed in *Y. pestis*. It is compatible with the *in vivo* mouse body
40 temperature and does not impact fitness of *Y. pestis*. The turnover production and activity of
41 the luciferase A and B subunits allows *in vivo* multiplication monitoring and correlates with
42 the bacterial load when a constitutive promoter is fused with the *lux* operon. The short half-
43 life of *P. luminescens* luciferase prevents signal accumulation and makes it appropriate for
44 quantitative studies.

45 In this chapter, we describe the use of bioluminescence for real-time *in vitro* and *in vivo*
46 monitoring of *Y. pestis* promoter activity. To allow the comparative study of gene expression
47 and to avoid side effects from plasmid variable copy numbers and plasmid loss *in vivo*, we
48 constructed a single chromosomal version of the *P. luminescens luxCDABE* operon fused
49 downstream of the *Y. pestis* promoter regions of interest. The mini Tn7 system was used for
50 specific chromosomal insertion at the *attTn7* site between two housekeeping genes, *glmS* and

51 *pstS*, encoding glucosamine-6-phosphate synthetase and periplasmic phosphate-binding
52 protein, respectively [4]. Transposition of mini Tn7 in this intergenic region does not modify
53 the virulence of *Y. pestis* [2].
54 Here we validate and evaluate the sensitivity of the bioluminescent reporter system using the
55 promoters of the *cafIM* and *YPO0499* genes, which are known to be regulated by temperature.
56 The *cafIM* gene is part of the *Y. pestis* F1 capsule encoding operon whose expression is up-
57 regulated at 37°C, while the gene *YPO0499* is part of operon encoding the type VI secretion
58 system whose expression is down-regulated at 37°C. The constitutively active promoter of
59 *rplN*, encoding a protein of the 50S ribosomal subunit was used as a control.

60

61 **2. Materials**

62 **2.1 Construction of *Y. pestis* reporter strains**

- 63 1. Fully virulent *Y. pestis* CO92 strain isolated from a fatal human pneumonic plague case [5]
64 (*see Note 1*).
- 65 2. *Escherichia coli* BW19610 strain [6].
- 66 3. pUC18R6KT mini-Tn7-*Km^r-luxCDABE* (**Fig. 1**) a non-replicative plasmid in *Yersinia* (*see*
67 **Note 2**), which requires a *pir* expressing strain, such as *Escherichia coli* BW19610, for its
68 replication.
- 69 4. Transposase-encoding plasmid pTNS2 [4] (**Fig. 1A**) (*see Note 3*).
- 70 5. Flp recombinase-encoding plasmid pFLP3 [4] (**Fig. 1B**).
- 71 6. Thermal Cycler
- 72 7. Primers for PCR amplification of *Y. pestis* promoter regions (*see Note 4*):
73 *PrpIN*, forward 5'-cgcACTAGTtagagtagccaactcgaacataataagc-3' and reverse 5'-
74 ataCCCGGGgctccgctaagtcaactac-3'; *PcafIM*, forward 5'-cgcACTAGTcttattttgctattcatcc-3'

75 and reverse 5'-tgaCCCGGGgcttgctattccttgctattcg-3'; *PYP00499*, forward 5'-
76 cgcACTAGTctattttgctattcatcc-3' and reverse 5'-tgaCCCGGGGcgttgctattccttgctattcgct-3'.
77 8. Primers for promoter sequence verifications: *PluxC* reverse 5'-cacgaatgtatgtctctgcgctc-3' and
78 *PKm* forward 5'-tcctatggaactgcctcggt-3'.

79 9. Primers for PCR verification of mini-Tn7 transposition: *PglmS* 5'-
80 gctatacgtgtttgctgatcaagatg-3', *PpstS* 5'- acgccaccggaagaaccgatacct-3', PTn7L 5'-
81 attagcttacgacgctacacc-3' and PTn7R 5'- cacagcataactggactgattc-3'.

82 10. Qiagen PCR purification kit

83 11. *SpeI* and *XmaI* endonuclease restriction enzymes

84 12. T4 DNA ligase

85 13. Sterile distilled water

86 14. Qiagen Midiprep plasmid purification kit.

87 15. Lysogeny broth medium (LB): 10 g tryptone, 5 g yeast extract and 10 g of sodium
88 chloride in 1 L of distilled water.

89 16. LB agar plates (LBA): 10 g tryptone, 5 g yeast extract, 10 g of sodium chloride and 15 g
90 of agar in 1 L of distilled water.

91 17. LBAH: LBA with 0.002% (w/v) hemin (*see Note 5*).

92 18. LBAHKm: LBAH with kanamycin (30 µg/mL).

93 19. LBAHCarb: LBAH with carbenicillin (100 µg/mL).

94 20. LBAHSuc: 10 g tryptone, 5 g yeast extract, 100 g sucrose, and 15 g of agar in 1 L of
95 distilled water.

96 21. Cold, sterile 10% glycerol.

97 22. 1X Phosphate buffer saline (PBS).

98 23. Electroporator

99 24. Sterile 0.2 cm electroporation cuvettes.

100 **2.2 *In vitro* measurement of promoter activity**

101 1. LB

102 2. PBS

103 3. 250 mL Erlenmeyer flask

104 2. Incubators set to 28°C and 37°C

105 3. Spectrophotometer and cuvettes

106 4. Plate reader capable for photon emission detection at 490 nm.

107 5. 96-well opaque white plate

108

109 **2.3 Measurement of promoter activity in a murine bubonic plague model**

110 1. 7 week-old female OF1 mice

111 2. Sterile 26G x 1/2" needles and 1 mL syringes

112 3. Sterile surgical dissection instruments

113 4. Tissue and cell disruption homogenizer (Tissuelyser II, Qiagen)

114 5. Sterile 2 mL polypropylene microtube with cap filled with 6 to 8 glass beads (3 mm

115 diameter, Omni international) for disruption of lymph nodes or 2 metal beads (2.4 mm, Omni

116 international) for skin disruption.

117 6. CO₂

118 7. Anesthetic mix in PBS: ketamine 20mg/mL (Imalgene 1000, Bayer) and xylazine 2mg/mL

119 (Rompun 2%, Merial).

120 8. Poly(methyl methacrylate) confinement box (TEM SEGA) (*see Note 6*).

121 9. Imaging System (IVIS Spectrum, Perkin Elmer)

122 10. Living Image 4.3.1 software (Caliper Life Sciences)

123

124 **3. Methods**

125

126 **3.1. Promoter amplification and cloning to generate a bioluminescent reporter plasmid**

127 **(Fig. 1A)**

128 1. Amplify the promoter region of interest using standard PCR methods with forward and
129 reverse primers for P^{rplN} , P^{caf1M} and $P^{YPO0499}$ regions.

130 2. Purify PCR products using the Qiagen PCR purification kit.

131 3. Digest the purified PCR product and the pUC18R6KT mini-Tn7- Km^r - $luxCDABE$ -based
132 vector with *SpeI* and *XmaI* following the enzyme manufacturer's instructions.

133 4. Ligate the digested PCR product and plasmid following the manufacturer's instructions
134 with T4 DNA ligase overnight at 16°C.

135 5. Electroporate 1-10 μ L of the ligation reaction into *Escherichia coli* BW19610
136 electrocompetent cells [7]. Add 1 mL of LB and incubate at 37°C for 45 min. Plate various
137 bacterial suspensions (pure to 1/100 dilution) on LBA supplemented with 100 μ g/mL
138 carbenicillin and incubate overnight at 37°C.

139 6. Check the correct insertion and sequence of the promoter of interest by restriction enzyme
140 digestion using *SpeI* and *XmaI* and by sequencing using primers *PKm* and *PluxC*. The
141 recombinant plasmid will be referred in this chapter as pUC18-mini-Tn7-K- P^{pestis} -*lux*.

142

143 **3.2 Construction of a single-copy bioluminescent reporter gene expression system in *Y.***

144 ***pestis* (Fig. 1)**

145 1. Purify plasmids pTNS2 (transposase provider) and pUC18-mini-Tn7-K- P^{pestis} -*lux* using the
146 Qiagen Midiprep plasmid purification kit.

147 2. Prepare electrocompetent *Y. pestis* CO92 cells resuspended in ice cold 10% glycerol (*see*
148 **Note 7**).

149 3. Electroporate 50 μ L of electrocompetent *Y. pestis* cells with 400 ng of each plasmid using
150 the following settings: 2.5 kV, 200 Ω and 25 μ F.

151 4. Add 1 mL of LB and incubate at 28°C under agitation for 90 min.

152 5. Plate various dilutions on LBAHKm and incubate for 48h at 28°C (recommended dilutions,
153 1/10 to 1/10³).

154 6. Verify the correct insertion of mini-Tn7-K-*P^{pestis}-lux* (**Fig. 1**) into the chromosomal *glmS*-
155 *pstS* intergenic region by PCR using the pairs of primers P*glmS*/PTn7R, P*pstS*/PTn7L and
156 P*glmS*/ P*pstS* flanking the *attTn7* insertion site (*see Note 8*).

157 7. Prepare *Y. pestis* CO92::*mini-Tn7-K-P^{pestis}-lux* electrocompetent cells resuspended in ice
158 cold 10% glycerol (*see Note 7*).

159 8. Remove the kanamycin resistant cassette by electroporation of 50 μ L electrocompetent *Y.*
160 *pestis* CO92::*mini-Tn7-K-P^{pestis}-lux* cells with 100 ng of plasmid pFLP3 (**Fig. 1B**).

161 9. Select the recombinant pFLP3 harboring cells by plating various dilutions on LBAHCarb
162 and incubate 48h at 28°C.

163 10. Replicate plate individual colonies on both LBAHKm and LBAH plates to identify the
164 clones that have lost the kanamycin resistance gene.

165 11. Eliminate the pFLP3 plasmid by growing *Y. pestis* CO92::*mini-Tn7-P^{pestis}-lux* on LB
166 overnight at 28°C.

167 12. Serial dilute the overnight culture and plate 100 μ L of 1/100 to 1/10⁶ dilutions on
168 LBAHSuc plates.

169 11. Replicate plate individual colonies on both LBAHCarb and LBAH to identify the clones
170 that have lost pFLP3.

171 12. Check for the presence of known unstable *Y. pestis* virulence genetic elements such as
172 plasmids, pYV and pPla and the High Pathogenicity Island by PCR [8] (*see Note 9*).

173

174 **3.3 *In Vitro* detection of promoter activity**

- 175 1. Make a lawn of *Y. pestis* recombinant strain (CO92 :: mini-Tn7-K- *P^{pestis}-lux*) by spreading
176 an aliquot from a -80°C frozen stock and incubating overnight at 28°C on LBAH.
- 177 2. Resuspend bacterial cells in fresh 10 mL LB by scraping from the bacterial lawn, adjust
178 optical density to 1, dilute 1/100 in 50 mL LB and grow under constant agitation (*see Note*
179 **10**). In this study two temperatures were tested, 28°C and 37°C (**Fig. 2**).
- 180 3. Measure the optical density at 600 nm over time using 1 mL samples (**Fig. 2**)
- 181 4. Measure the photon emission per second (RLU) over time by transferring 0.1 mL sample
182 from the 50 mL growth in a 96 well plate (**Fig. 2**) (*see Note 11*).
- 183 5. To determine promoter activity, divide the number of photon/sec (RLU) by the absorbance
184 at 600 nm in exponential phase to correct for the relative differences in cell number between
185 strains.

186

187 **3.4 *In vivo* detection of *Y. pestis* promoter activity in a murine bubonic plague model**

188 Animal experiments must receive approval from national and institutional committees.

189 Our protocol was approved by the French committee CETEA (n°2014-0025) and Institut

190 Pasteur CHSCT (n°0399).

- 191 1. Grow bioluminescent *Y. pestis* on LBAH plates overnight at 28°C, as described in section
192 3.3.1.
- 193 2. Resuspend bacteria in PBS and adjust OD600 to 0.1, as described in section 3.3.2.
- 194 3. Prepare serial dilutions in PBS.
- 195 4. Inject the required amounts of bacterial cells within a maximal volume of 50 µL for
196 subcutaneous injection (*see Note 12*). In the experiment shown in figure 3, 10e5 CFU were
197 injected (*see Note 13*)

198 5. At desired time points, anesthetize the mice by injecting intraperitoneally 100 μ l of the
199 anesthetic mix (ketamine/xylazine).

200 6. Place mice in a confinement box (*see Note 6*).

201 7. Place the confinement box into the IVIS imaging chamber with the mice injection site
202 facing the CCD camera.

203 8. Choose the field of view according to the number of animals to be imaged (*see Note 14*).

204 9. Set up the imaging acquisition conditions on the control panel by selecting luminescent
205 imaging mode, block excitation filter and open emission filter.

206 10. Adjust the binning to “small” and the exposure time according to the intensity of the
207 signal (between 5 sec to 2 min exposure time) (*see Note 15*).

208 11. Click “acquire” to measure luminescence (**Fig. 3**).

209 12. Use the tool palette/image adjust to adjust the color scale in order to visualize all sites that
210 exhibit signals above the background (*see Note 16*).

211 13. Create regions of interest (ROI) by using the ROI tool (*see Note 17*) and measure ROIs.

212 14. In order to evaluate the background bioluminescent signal, measure the number of
213 photons emitted from the same ROI of a non-infected mouse placed in the same confinement
214 box as infected mice (*see Note 16*).

215 15. Calculate the luminescence to determine changes in luciferase activity over time.
216 Promoter activity is measured by counting the photons emitted by the bacteria. To allow
217 comparisons between signals express the ROI bioluminescence in average radiance
218 (photons/sec/cm²/steradian).

219 13. To determine the number of bacteria in the infected tissues, euthanize mice with CO₂.

220 14. Harvest the tissues aseptically and place them in a 0.5 mL sterile PBS tubes containing
221 glass or metal beads.

- 222 15. Apply a 2 min tissue disruption cycle of 30 agitations/sec. with the TissueLyser II Qiagen
223 apparatus (*see Note 18*)
- 224 16. Spread 50 μ L of serial dilutions of the cell suspension on LBAH plates and incubate 48h
225 at 28°C for colony counts.
- 226 17. Results are given in ROI per CFU (**Fig. 3B**).

227

228 4. Notes

- 229 1. Experiments performed on fully virulent *Y. pestis* require a Biosafety Level 3 laboratory
230 and animal facility.
- 231 2. The plasmid pUC18R6KT mini-Tn7-*Km^r-luxCDABE* was constructed by subcloning (i) the
232 *Km* resistance cassette from (*Km^r*) pFKM1[4] into the *SacI* restriction site and (ii) the
233 *P. luminescens luxCDABE* operon into the *ApaI/XmaI* restriction sites of pUC18R6KT mini-
234 Tn7 [4]. The resulting plasmid does not contain any promoter region for *luxCDABE*
235 expression and is used for evaluation of bioluminescence background in *in vitro* studies, as
236 well as for subsequent cloning of the different *Y. pestis* promoters to be studied.
- 237 3. Plasmid pTNS2 is not replicative in *Y. pestis*. Efficient production of the transposase
238 immediately after pTNS2 introduction in *Y. pestis* allows transposition of the mini Tn7. Loss
239 of the transposase encoding plasmid will prevent subsequent transposition of Tn7.
240 Verification of the loss of pTNS2 after Tn7 transposition in *Y. pestis* chromosome is
241 performed by testing carbenicillin susceptibility on LBAHCarb.
- 242 4. For a directional cloning of promoter regions upstream of the *luxCDABE* operon, forward
243 and reverse primers contain 5' *SpeI* and *XmaI* restriction sites, respectively (**Fig. 1A**).
- 244 5. When colony forming units (CFU) count have to be performed on *Y. pestis* suspension it is
245 recommended to supplement the agar lysogeny broth medium with 0.002% (w/v) porcine
246 hemin. The presence of hemin in the medium will allow individual bacteria to grow and form

247 a CFU within 48 hours at 28°C. If hemin is omitted colonies will be small, difficult to count
248 and the number of colonies could be underestimated.

249 6. When animals are infected with a class 3 pathogen, their transportation and visualization in
250 the IVIS spectrum system should be conducted in a biosafety containment box. In our study
251 we used a Poly(methyl methacrylate) confinement box (TEM SEGA) to warrant the
252 confinement of infected animals and to allow the photon acquisition by the CCD detector.

253 7. To prepare electrocompetent *Y. pestis*, bacteria were grown overnight at 28°C in LB. The
254 culture was diluted (1/50) in fresh broth and grown until reaching A600=0.8. Bacterial cells
255 were harvested by centrifugation (4000xg, 15 min, 4°C), washed twice in cold sterile distilled
256 water and once in 10% glycerol. The pellet was resuspended in 10% glycerol to reach a
257 concentration factor of 500 times compared to the original growth volume. Electrocompetent
258 cells were aliquoted in 50 μ L and frozen at -80°C until use.

259 8. Correct insertion of the mini-Tn7 transposon is verified by the amplification of 770 bp
260 and 336 bp PCR fragments with primers *PpstS*/*PTn7L* and *PglmS*/*PpstS* respectively and
261 absence of amplification with primers *glmS-pstS* when the following standard PCR program
262 is used: 1 cycle at 95°C for 3 min, 35 cycles (i) at 95°C for 30 sec , (ii) at 55°C for 30 sec,
263 and (iii) at 72°C for 1 min and finally 1 cycle at 72°C for 3 min.

264 9. Presence of the pYV and pPla plasmids and the high pathogenicity island are verified by
265 PCR amplifications of 565 bp *yopM*, 480 bp *pla* and 434 bp *irp2* fragments using primer pairs
266 *YopM-f* (5'-ataactcatcgggggcaaaat-3')/*YopM-r* (5'-gcgttattatccgaatttagc-3'), *pla-f* (5'-
267 atcttactttccgtgagaag-3')/*pla-r* (5'-cttgatgttgagcttccta-3') and *irp2-f* (5'-tgatgagatggttacactgg-
268 3')/*irp2-r* (5'-gtcgttatggcgtaaatcat-3'), respectively. The standard PCR program indicated in
269 Note 8 is used.

270 10. Conditions of growth culture may vary depending on the known or putative activity of the
271 promoter region studied.

272 11. Since *Y. pestis* tends to aggregate when grown in LB, we recommend to perform the in
273 vitro growth in a large volume (50 mL LB) in a 250 mL Erlenmeyer flask under 140 rpm
274 agitation and measure the absorbance overtime by taking 0.1 mL samples.

275 12. To facilitate the subcutaneous injection, the fur of the animal can be locally shaved 24h
276 before the injection. Depending the site of injection anesthesia can be required prior to the
277 injection. In the experiment shown in figure 3 the animals were not anesthetized.

278 13. The volume and dose injected can vary depending on the injection mode, site and the
279 expected level of signal at early time points of the infection.

280 14. IVIS spectrum allows the use of different fields of view. When more than three animals
281 are imaged use field of view D. Sensitivity of the detection camera may vary depending on
282 the field of view chosen during acquisition. If a comparative study is performed make sure to
283 use the same field of view throughout the experiment.

284 15. For quantification, make sure to use an exposure time that does not lead to signal
285 saturation. In the image display, a message indicates saturated signals. In most of our
286 experiments the exposure time varied from 5 to 240 sec with a small binning. If a weak signal
287 is expected, the exposure time can be increased up to 5 min with a large binning.

288 16. When several infected animals are to be imaged within the same containment box, it is
289 important to evaluate the level of the background signal by imaging a non-infected animal
290 hosted in the same box. This level may change depending on the intensity of the overall signal
291 within the same box.

292 17. The shape and size of the ROI will be adjusted to the shape and size of the site exhibiting
293 a bioluminescent signal. In the case of the site of injection shown in figure 3, the shape and
294 size of the ROI were drawn by using the function "duplicate".

295 18. For tissue disruption, other homogenizer apparatuses can be used. If so, validation of the
296 efficiency of bacterial count must be performed.

297

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300 Photonic BioImaging facility of the Institut Pasteur.

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- 319
- 320

321 **Figure Legends**

322 **Fig. 1** Construction of the bioluminescent reporter vector to study *Y. pestis* promoter activity.

323 (A) Suicide delivery pUCR6K-mini Tn7 based vector. Tn7L and Tn7R, left and right ends of
324 Tn7; T₀T₁, transcriptional terminators; *bla*, β-lactamase-encoding gene; Km^R kanamycin
325 resistance encoding cassette flanked by FRT, Flp recombinase target; P^{pestis}, *Y. pestis*
326 promoter regions; *luxCDABE*, *Photobacterium luminescens lux* operon where *luxA* and *luxB*
327 encode the subunits A and B of bacterial luciferase, while *luxC*, *luxD*, and *luxE* encode the
328 subunits of a fatty acid reductase that provides the fatty aldehydes to the luciferase. Only
329 enzyme restriction sites used for cloning Km^R, promoter regions and *lux* operon are indicated.
330 pTNS2, helper suicide plasmid encoding the specific TnsABC+D transposition pathway.
331 Transposition of mini Tn7-Km^R-P^{pestis}-*lux* into *Y. pestis* chromosome is achieved by co-
332 electroporation of competent *Y. pestis* CO92 cells with pTNS2 and pUCR6K-KmR-*lux*.
333 *attTn7* unique insertion site located in the 482-bp *glmS*-*pstS* intergenic region.

334 (B) Removal of the Km^R cassette. Mini Tn7-Km^R-P^{pestis}-*lux* reporter system integrated as a
335 monocopy in *Y. pestis* chromosome; pFLP3, Flp recombinase encoding plasmid. Plasmid
336 pFLP3 is electroporated into the bioluminescent *Y. pestis* CO92 recombinant strain to remove
337 the KmR cassette in order to avoid any transcriptional interference with the bioluminescence
338 reporter system.

339
340 **Fig. 2** Detection of bioluminescence *in vitro*.

341 *Yersinia pestis* CO92 recombinant strains were pre-grown on LBAH plate. Then bacterial
342 cells were resuspended in PBS, optical density at 600 nm was adjusted and a 50 mL LB flask
343 was inoculated 1/100e to obtain approximately 10⁷ CFU/mL and incubated under agitation.
344 Optical density and photon emission were recorded overtime by taking a 1 mL and 0.1 mL
345 aliquot of the growth suspension, respectively. (A) Detection of bioluminescence signals
346 obtained with strains CO2:: Tn7-P^{less}-*lux* and Tn7-P^{rrpIN}-*lux* when grown in LB at 28°C and

347 37°C. The CO92::Tn7-P^{less}-*lux* strain was used as a negative control to evaluate in each
348 growth condition the bioluminescence background. The promoter region of the *rplN*
349 ribosomal encoding gene was used as a constitutively active promoter in each growth
350 condition. Since the ratio RLU over CFU did not vary significantly over time nor with
351 temperature for the two control strains, only an average of the signals measured in each
352 growth condition are indicated. Detection of *Y. pestis* P^{caf} (B) and P^{YPO0499} (C) activities, two
353 temperature sensitive promoters in LB at 28°C and 37°C. The increase of bioluminescence
354 reflects the activity of the P^{caf} promoter showing upregulated *Y. pestis* capsule expression,
355 while the decrease of bioluminescence reflects the downregulated expression of the type VI
356 secretion *YPO0499* gene at 37°C. Statistical analyses were performed with an unpaired t-test
357 (p<0,0001 ****, p<0,0003 ***, p<0,001 **) using Prism 5 GraphPad software.

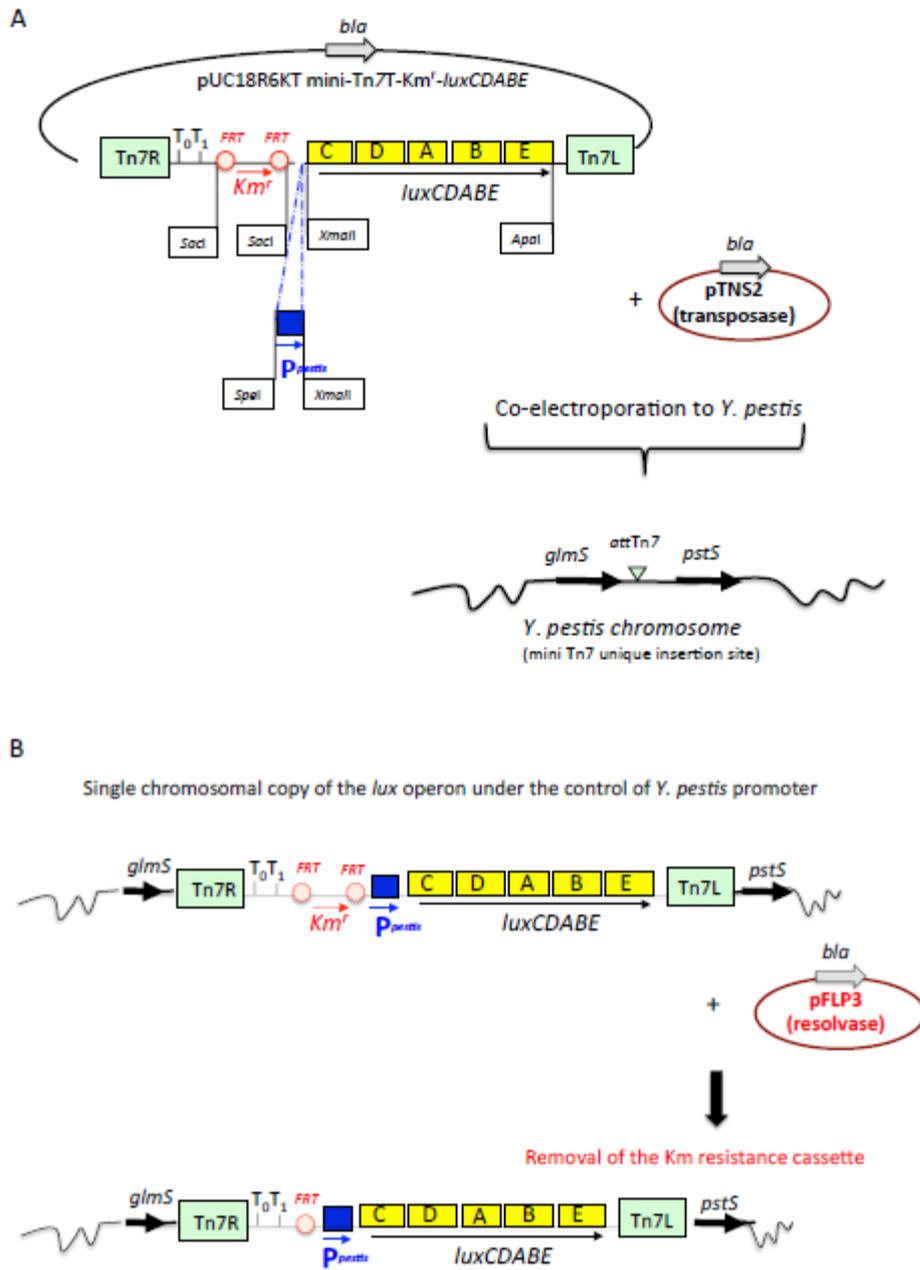
358

359 **Fig. 3** Bioluminescence imaging of *Y. pestis* infected mice.

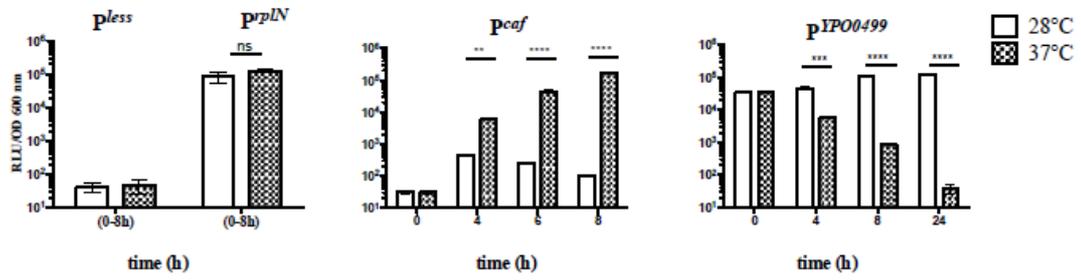
360 (A) OF1 mice were infected subcutaneously in the linea alba with 10e5 CFU of *Y. pestis*
361 CO92::Tn7-P^{rplN}-*lux* or P^{YPO0499}-*lux* and imaged at different time points using an IVIS
362 Spectrum imaging system. Sequential images from representative animals are shown. The
363 color scales represent the light emission and is kept constant throughout the observation
364 period. In this study a small binning and an acquisition time of 10s to 240s were used.
365 Uninfected mice (UI) were used to set the light emission background. Regions of interest
366 (ROI) were drawn using the Living Image 4.3.1 software and average bioluminescence
367 (photon/sec/cm2/sr) was calculated for the injection site, as shown here for the P^{rplN}-fusion at
368 0.5h post injection in the skin. Bioluminescence signal is detected in the skin at the site of
369 injection. The increased signal observed with the P^{rplN}-*lux* fusion construct reflects *Y. pestis*
370 multiplication. In addition, bacterial dissemination to the inguinal and axillary draining lymph
371 nodes is detected 44h post infection.

372 (B) Evaluation of promoter activity during *Y. pestis* multiplication in the skin of OF1 mice.
373 Mice were sacrificed at indicated time points. The site of injection was dissected and the skin
374 was homogenized to determine bacterial loads (CFU) and compare them to the measured ROI
375 (photon/sec/cm²/sr). P^{*rpIN*}-*lux* fusion illustrates the constitutive expression of *rpIN*, where the
376 ROI/CFU ratio is similar over the time. The P^{*YPO0499*}-*lux* fusion demonstrates the decrease of
377 *YPO0499* expression when the pathogen multiplies in the skin as exemplified by the decrease
378 ROI/CFU ratio.

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418 Figure 2



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