



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

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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

6 Running title

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

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.

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

1. Introduction

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

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

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

pstS, encoding glucosamine-6-phosphate synthetase and periplasmic phosphate-binding protein, respectively [4]. Transposition of mini Tn7 in this intergenic region does not modify the virulence of *Y. pestis* [2].

Here we validate and evaluate the sensitivity of the bioluminescent reporter system using the promoters of the *cafIM* and *YPO0499* genes, which are known to be regulated by temperature. The *cafIM* gene is part of the *Y. pestis* F1 capsule encoding operon whose expression is up-regulated at 37°C, while the gene *YPO0499* is part of operon encoding the type VI secretion system whose expression is down-regulated at 37°C. The constitutively active promoter of *rplN*, encoding a protein of the 50S ribosomal subunit was used as a control.

2. Materials

2.1 Construction of *Y. pestis* reporter strains

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

75 and reverse 5'-tgaCCCGGGgcttgctattccttgctattcg-3'; *PYP00499*, forward 5'-
 76 cgcACTAGTctattttggctattcatcc-3' and reverse 5'-tgaCCCGGGGcgttgctattccttgctattcgct-3'.
 77 8. Primers for promoter sequence verifications: *PhxC* reverse 5'-cacgaatgtatgtcctgcgtc-3' and
 78 *PKm* forward 5'-tcctatggaactgcctcgt-3'.
 79 9. Primers for PCR verification of mini-Tn7 transposition: *PglmS* 5'-
 80 gctatacgtgttgctgatcaagatg-3', *PpstS* 5'- acgccaccggaagaaccgatacct-3', PTn7L 5'-
 81 attagcttacgacgtacaccc-3' and PTn7R 5'- cacagcataactggactgatttc-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**

3.1. Promoter amplification and cloning to generate a bioluminescent reporter plasmid (Fig. 1A)

1. Amplify the promoter region of interest using standard PCR methods with forward and reverse primers for P^{rplN} , P^{caf1M} and $P^{YPO0499}$ regions.
2. Purify PCR products using the Qiagen PCR purification kit.
3. Digest the purified PCR product and the pUC18R6KT mini-Tn7- Km^r - $luxCDABE$ -based vector with *SpeI* and *XmaI* following the enzyme manufacturer's instructions.
4. Ligate the digested PCR product and plasmid following the manufacturer's instructions with T4 DNA ligase overnight at 16°C.
5. Electroporate 1-10 µL of the ligation reaction into *Escherichia coli* BW19610 electrocompetent cells [7]. Add 1 mL of LB and incubate at 37°C for 45 min. Plate various bacterial suspensions (pure to 1/100 dilution) on LBA supplemented with 100 µg/mL carbenicillin and incubate overnight at 37°C.
6. Check the correct insertion and sequence of the promoter of interest by restriction enzyme digestion using *SpeI* and *XmaI* and by sequencing using primers *PKm* and *PluxC*. The recombinant plasmid will be referred in this chapter as pUC18-mini-Tn7-K- P^{pestis} - lux .

3.2 Construction of a single-copy bioluminescent reporter gene expression system in *Y. pestis* (Fig. 1)

1. Purify plasmids pTNS2 (transposase provider) and pUC18-mini-Tn7-K- P^{pestis} - lux using the Qiagen Midiprep plasmid purification kit.
2. Prepare electrocompetent *Y. pestis* CO92 cells resuspended in ice cold 10% glycerol (see 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 *PglmS*/PTn7R, *PpstS*/PTn7L and
156 *PglmS*/ *PpstS* 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

3.3 *In Vitro* detection of promoter activity

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

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

Animal experiments must receive approval from national and institutional committees.

Our protocol was approved by the French committee CETEA (n°2014-0025) and Institut Pasteur CHSCT (n°0399).

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

198 5. At desired time points, anesthetize the mice by injecting intraperitoneally 100 µl 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.

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

4. Notes

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

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

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

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

8. Correct insertion of the mini-Tn7 transposon is verified by the amplification of 770 bp and 336 bp PCR fragments with primers *PpstS*/PTn7L and *PglmS*/*PpstS* respectively and absence of amplification with primers *glmS-pstS* when the following standard PCR program 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, and (iii) at 72°C for 1 min and finally 1 cycle at 72°C for 3 min.

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

10. Conditions of growth culture may vary depending on the known or putative activity of the 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

Figure Legends

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

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

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

Fig. 2 Detection of bioluminescence *in vitro*.

Yersinia pestis CO92 recombinant strains were pre-grown on LBAH plate. Then bacterial cells were resuspended in PBS, optical density at 600 nm was adjusted and a 50 mL LB flask was inoculated 1/100e to obtain approximately 10e7 CFU/mL and incubated under agitation. Optical density and photon emission were recorded overtime by taking a 1 mL and 0.1 mL aliquot of the growth suspension, respectively. (A) Detection of bioluminescence signals obtained with strains CO2:: Tn7-P^{less}-*lux* and Tn7-P^{prpIN}-*lux* when grown in LB at 28°C and

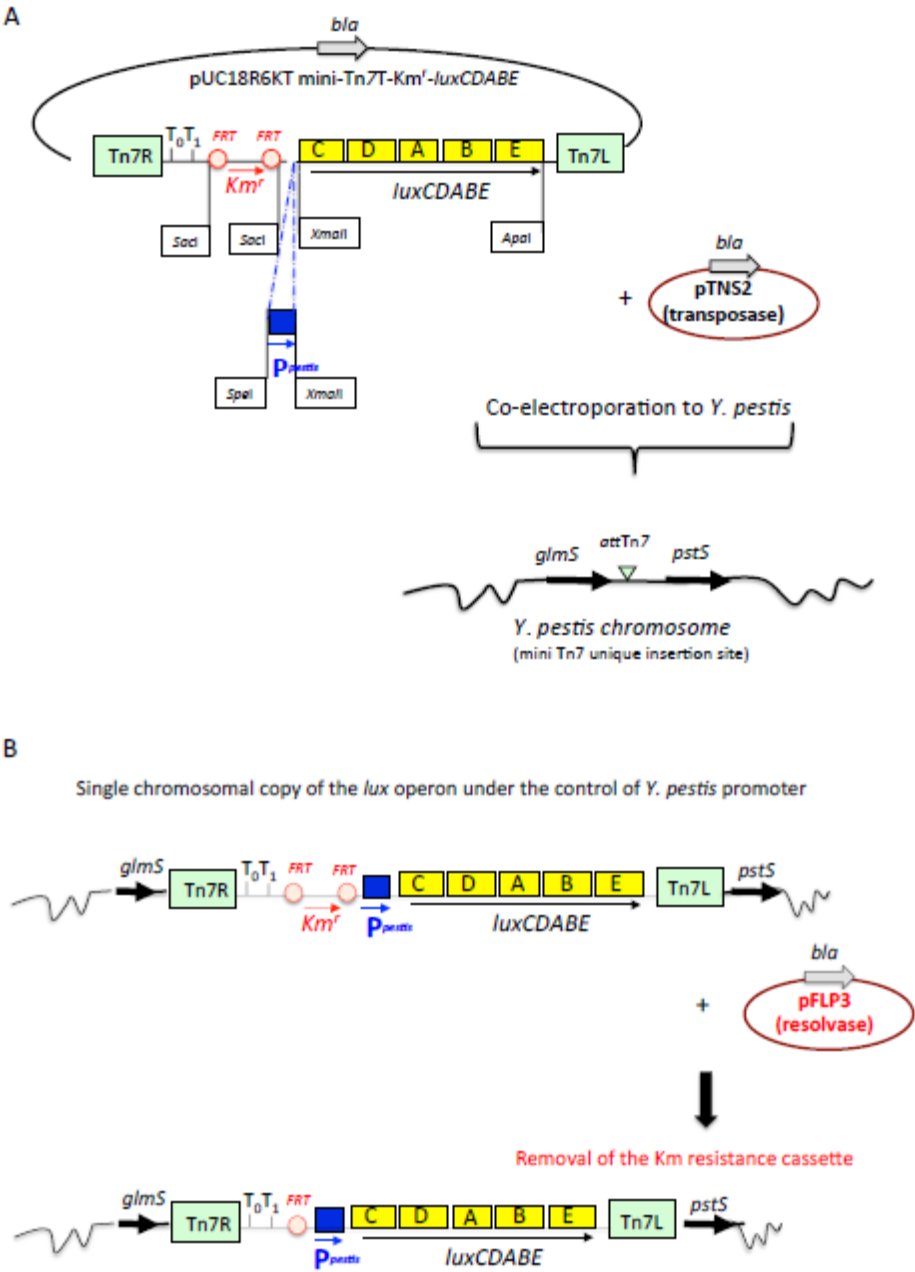
37°C. The CO92::Tn7-P^{less}-*lux* strain was used as a negative control to evaluate in each growth condition the bioluminescence background. The promoter region of the *rplN* ribosomal encoding gene was used as a constitutively active promoter in each growth condition. Since the ratio RLU over CFU did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

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

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

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

415 Figure 1



416

417

418 Figure 2

