Cationic antimicrobial peptide resistant sub-population in *P. luminescens* TT01 strain is responsible for virulence in insects

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Introduction

Bacteria live in environments that undergo perpetual alterations and in which they are challenged by antibiotics, bacteriophages, mutagens, toxins and more. Which strategies do bacteria use to optimize their chance of surviving? Among mechanisms used by bacteria to survive, there are two key strategies: phase and antigenic variation (Van der Woude 2011) that corresponds to genetic alterations and the bistability generated by epigenetic mechanisms on clonal population (Dubnau et Losick 2006). Both of these strategies lead to bacterial heterogeneity. Bacterial population has been traditionally seen as an isogenic and clonal population genetically and phenotipically identical. The development of single cell technology such as cytometry and fluorescence microscopy allowed the development of studies showing heterogeneous gene expression in bacterial cells (Smits, Kuipers, et Veening 2006). Heterogeneity is found in various bacteria like *Salmonella* in *Salmonella*-containing vacuoles (Helaine et Holden 2013) or *Photorhabdus* during the colonization of nematodes (Somvanshi *et al*. 2012).

Photorhabdus luminescens subsp laumondii TT01 is an entomopathogenic bacterium (Enterobacteriaceae) living in a symbiotic association with the nematode *Heterorhabditis*. The bacteria-nematode complex invades insect larvae and the nematode regurgitates its bacterial symbiont directly into the hemolymph, the insect blood. The bacteria can overcome the insect immune system and colonize the insect body cavity leading to lethal septicemia (Waterfield, Ciche, and Clarke 2009). Bacterial virulence factors and insecticidal toxins also participate to the insect death (Silva *et al*. 2002 ; Nielsen-LeRoux *et al*. 2012). Once the insect host is dead, bacteria bioconvert the tissues, digest the content of the cadaver and the nematode feeds on it as a food source while reproduction occurs through several generations (Clarke 2014). According to its dual lifestyle, *Photorhabdus* is a good model to study bacteria-insects and bacteria-nematode interactions. Recently, it has been demonstrated that the *mad* genes expression is under the control of a genetic switch of the promoter region. In the ON state *mad* genes can be transcribed and the bacteria are covered with fimbriae. This form of *Photorhabdus* is called the M form (for mutualistic form) because it is found only during the symbiosis with the nematode. But, when the *mad* promoter is in the OFF conformation, no fimbriae are produced on the bacterial cell surface and only the P form (for pathogenic form) of *Photorhabdus* is found. Only the P form, can multiply and kill the insect and can support nematode growth (Somvanshi *et al*. 2012).

Cationic antimicrobial peptides (CAMPs) are produced following insect infection (Bang et al. 2012 ; Haine *et al*. 2008) and act in complement of cellular immunity to fight against bacterial invasion. CAMPs are small amphipathic basic peptides from 15-40 amino acids (for review see (Bulet and Stöcklin 2005)) secreted by a large number of organisms including plants, animals and microbes and present a large variety of structure. However, the two prominent classes involved alpha-helical and beta-sheet peptides (Tossi, Sandri, and Giangaspero 2000). CAMPs have antibacterial activity acting through charge interactions with the anionic bacterial surface, predominantly binding to the acidic lipid A moiety of the LPS (Rana *et al*. 1991 ; Srimal *et al*. 1996).

PhoPQ has been extensively studied in *Salmonella* where it controls about 3% of *Salmonella* gene expression either in a direct or indirect pathway (Kato, Groisman, and Howard Hughes Medical Institute 2008). Among genes regulated by PhoPQ, virulence factors or genes implicated in LPS modifications are found such as *pagP* and *pbgPE*. *pagP* is directly regulated by PhoP in *Salmonella* spp, and responsible for addition of palmitate residue on lipid A of the LPS. This lipid A palmitoylation confers resistance towards cationic antimicrobial peptides (CAMPs) (Guo *et al*. 1998) and reduce bacterial recognition by immune system in a TLR4 dependant pathway (Kawasaki, Ernst, et Miller 2004). Another modification involved in LPS modification is the addition of an amino-arabinose on lipid A core of the LPS (Gunn et Miller 1996). This modification modifies the global net charge of bacterial cell membrane from negative to positive conferring resistance towards CAMPs (Gunn *et al*. 1998). In *Salmonella* the *pbgPE* operon codes for enzymes responsible for amino-arabinose addition on lipid A. PhoP indirectly regulates *pbgPE* expression via another two component system PmrAB (Gunn et Miller 1996). *pbgPE* expression is activated at low Mg^{2+} concentrations. *pbgPE* has been shown to have a role in bacterial virulence and homologues have been described in other Gram negative bacteria such as *Yersinia*, *E. coli* and *Photorhabdus*. *pbgPE* operon in *Photorhabdus* is also required for virulence in insects (Bennett et Clarke 2005) but neither *pmrAB* nor *pmrD* genes were found in *P.luminescens* genome (Duchaud *et al*. 2003). In addition, PhoP-PhoQ also plays an essential role in virulence phenotype in that *phoP* mutant lead to completely avirulent phenotype in lepidopteran insects and sensibility towards CAMPs such as polymyxin B and cecropins A and B (Derzelle *et al*. 2004). In addition, we recently showed that $ail1_{Pl}$ gene encoding an OMP belonging to the Ail/OmpX/PagC/Lom family was directly regulated by PhoP. $ail1_{Pl}$ also respond to Mg^{2+}, the main *in vitro* inductor, responsible for PhoPQ activation as reported in *Salmonella* (Mouammine *et al*. 2014).

Here, we demonstrated that the virulence strategy of *P. luminescens* is to produce a stable sub-population resistant towards CAMPs which is responsible of septicemia in insects. The resistant bacteria represent only 0.5% of the wild type population in *P. luminescens* TT01 during in vitro cultures and we demonstrated that this heterogeneity relies on PhoP and *pbgPE*. Heterogeneity appears to be a key mechanism for *Photorhabdus* life cycle.

Material and Methods

Bacterial strains, plasmids, and growth conditions The strains and plasmids used in this study are listed in Table S1. *P. luminescens* strains were routinely grown at 28°C in Luria-Bertani (LB) or Mueller Hinton broth (Biokar), nutrient agar medium (Difco), NBTA agar (Brunel *et al*. 1997). *Photorhabdus* was also grown in M9 liquid medium supplemented with 0.1 % casamino acids, 0.41 mM nicotinic acid, 9.1 mM 1sodium pyruvate, 0.1 mM CaCl2 and 0.2 % glycerol with different concentrations of $MgSO_4$ (10 µM and 10 mM). When required, antibiotics were used at the following final concentrations: polymyxin B 100 $mg.l^{-1}$, kanamycin, 20 mg.l-^{1}, gentamicin, 15 $mg.l^{-1}$, erythromycin 15 $mg.l^{-1}$.

Antibacterial activity.

In vitro susceptibility tests to determine MICs were performed by the broth microdilution method according to National Committee for Clinical Laboratory Standards proposed guidelines (Hetru et Bulet 1997), with some modifications. Stock solutions of colistin methane sulfonate (Sigma) and polymyxin B (Sigma) were diluted in sterile water to obtain concentrations of 20 and 50 mg/ml, respectively. Stock solutions of cecropin A and B were prepared in 0.5% acetic acid to obtain a concentration of 0.4 mg/ml, cecropin A from *S. frugiperda* was prepared in distilled water at 0.5 mg/ml. Antibiotics were then added directly to 96-well microtiter plates in twofold serial dilutions. 10^4 bacteria grown at a OD 0.6-0.8 was dispensed into each microdilution well. The MICs were determined in Mueller-

Hinton broth (Biokar) following incubation at 28°C for 48 h. The microtiter plates were read by visual observation.

Protein PhoP-His purification protocol

The entire coding region of *phoP* gene from TT01 strain was amplified by PCR and digested by *Nde*I and *Bam*HI (Table S2). The ligation of the PCR product obtained was performed into the same site of the expression vector, pETPhos (Pfaffl, Horgan, et Dempfle 2002) inserting a His-tag in N-term part of proteins thereby generating P_{T7}PhoP-His. The recombinant plasmid encoding a PhoP-His fusion protein was transformed into *E. coli* BL21 (DE3) pLysS cells. At an OD between 0.5-0.8, the expression of PhoP-His was induced by adding isopropyl-beta-D-thiogalactoside at 0.5 mM, then an overnight induction was performed at 18°C. Bacterial culture was centrifuged at *7,000 x g* for 15 min *at* 4°C, washed twice in resuspension buffer (Tris 5 mM pH 7.5, NaCl 300 mM, Glycerol 10 %, Imidazole 10 mM) and pellet was frozen at -80°C for 30 min. Pellet was then suspended in 5 ml resuspension buffer and lysed by sonication during 10 min at 4°C. Lysis products were centrifuged at 10,000 x *g* during 30 min at 4°C. 500 µL of pre-equilibrated beads of Ni-NTA agarose (Qiagen) in the wash buffer (Tris 5 mM pH 7.5, NaCl 300 mM, glycerol 10 %, Imidazole 15 mM) were added to the supernatant fraction and incubated during 45 min with shaking at 4°C. The fraction was centrifuged at 500 x *g* during 2 min at 4°C and wash 5 times with wash buffer. Protein was eluted twice in 1 mL elution buffer (Tris 5 mM pH 7.5, NaCl 300 mM, glycerol 10%, Imidazole 200 mM). Concentration of recombinant protein was assessed by Bradford assay and controlled by SDS-page gel. Recombinant proteins were conserved at -80°C until use.

Electrophoretic mobility-shift assays (EMSA)

The promoter of $ail1_{Pl}$ was amplified by PCR from the genomic DNA of TT01 strain using primers (Table S2) and purified using the High Pure PCR Product Purification kit (ROCHE).

The 5' ends of DNA were labeled using [γ-^{32}P] ATP and T4 polynucleotide kinase (Promega). Radioactive DNA probe (2000 cpm/ml), 200ng of poly(dI-dC)-poly(dI-dC) (SIGMA) and different amounts of PhoP-His were mixed with binding buffer (50 mM tris-HCl pH 8, 50 mM KCl, 50 µg/mL BSA) in a total 20µl volume and incubated for 20 min at room

temperature. The mixture was then loaded onto a native 6 % (w/v) polyacrylamide TBE precast Gel (Invitrogen) and electrophoresed in 1 % TBE (Tris-Borate-EDTA) buffer for 1 h at 100 V. Radioactive species were detected by autoradiography. PhoP-His was activated by in vitro phosphorylation with acetyl phosphate as previously described (Jubelin *et al*. 2013).

Molecular techniques and RNA preparation

DNA manipulations were carried out as previously described (Ausubel *et al*. 1999). Plasmids were introduced into *E. coli* Wm3064 (Table S1) by transformation and transferred to *P. luminescens* by filter mating (Brillard *et al*. 2002). All constructs were sequenced by MWG operon Eurofins. Total RNA was extracted and purified with the RNeasy miniprep kit (Qiagen), including a DNase I treatment step. For each RNA preparation, we assessed DNA contamination by carrying out a control PCR. The quantity and quality of RNA, respectively, were assessed with a NanoDrop 2000 spectrophotometer (Thermo Scientific) and an Agilent 2100 Bioanalyzer with the RNA 6000 Nano LabChip kit (Agilent). Material for qPCR analysis was prepared by extracting total RNA from the *P. luminescens* wild-type strain grown in Luria broth, in Luria broth supplemented with polymyxin B or M9 medium supplemented with 10 µM or 10 mM MgSO4. For the comparison between total and resistant population, RNA were prepared from an OD540=0.3 culture before adding polymyxin B in the medium and after adding polymyxin B (around 10-12 hours later when the OD reach again 0.3). Samples were differentially analyzed to evaluate gene expression level before and after adding polymyxin B (three independent biological replicates) and in 10 µM versus 10 mM MgSO4 M9 supplemented medium. The primers used in this study (Eurogentec) are described in Table S2.

RT-qPCR analysis

RT-qPCR was performed in two steps. First, the cDNA was synthesized from 500 ng of total RNA, with Super Script II Reverse Transcriptase (Invitrogen) and random hexamers (100 ng/µl) (Applied Biosystems). We then carried out qPCR in triplicate with the LightCycler 480 SYBR Green I Master kit from Roche Diagnostics, with 1 µl of cDNA synthesis mixture (diluted 1:100) and 1 µM of specific primers for the genes studied (Table S2). The enzyme was activated by heating for 10 min at 95°C. All qPCRs were performed in

three technical replicates, with 45 cycles of 95°C for 5 seconds, 60°C for 5 seconds and 72°C for 10 seconds, and were monitored with the LightCycler 480 system (Roche). Melting curves were analyzed for each reaction and each curve contained a single peak. The data for each sample are expressed relative to the expression level of *gyr*, using REST software 2009 (Pfaffl, Horgan, et Dempfle 2002) as previously described in (Jubelin *et al*. 2013). This method provided a relative quantification of the expression of a target gene with respect to a reference gene, for the comparison of the wild-type strain in different growth conditions.

Evaluation of resistant sub-population.

Antibiograms are performed as follows. An exponentially phase culture was diluted in Mueller-Hinton medium and 1 mL of a total of 10^3 CFU was spread on Mueller Hinton agar plates and lay for 5 minutes. The excess was removed and plates were let to dry for 5 to 10 minutes. Paper discs were filed on the plates on which 500 µg and 50 µg of polymyxin B were added in a maximal volume of 10 µl. Plates were incubated at 28°C and results were observed after 48h incubation.

To quantify the proportion of resistant sub-population in total population *in vitro*, we assessed CFU on nutrient agar plates. Samples of the same culture of wild-type strains were diluted and at least three dilutions were spread on plates with nutrient agar or nutrient agar supplemented with polymyxin B 100µg.mL^{-1} final concentration to isolate the resistant sub-population. Samples were collected during bacterial growth at each key point (lag, exponential and stationary phase). Plates were counted 48 h after incubation at 28°C.

Construction of plasmids expressing *gfp*[AAV] under the control of *pbgPE* gene promoter .

As described in (Jubelin *et al*. 2011), we use a similar method to construct plasmids expressing the reporter gene *gfp*[AAV] under the control of the *pbgPE or lac* promoter region. The construction of P$_{lac}$-*gfp*[AAV] has been described elsewhere (Abi Khattar 2009). The construction of the P$_{pbgPE}$-*gfp*[AAV] was performed as follows. Briefly, DNA fragment corresponding to the *pbgPE* promoter region (198-bp) was amplified by PCR from TT01 genomic DNA with primers containing a *Kpn*I or *Xba*I restriction site. The PCR products were digested and inserted into the corresponding sites of pPROBE-*gfp*[AAV] plasmid.

Finally, P_{lac}-*gfp*[AAV] and P_{pbgPE}-*gfp*[AAV] were transferred by bacterial mating in TT01 strain.

pbgPE expression in individual bacterial cells by flow cytometry

Bacterial strains were grown in LB supplemented with kanamycin at 28°C. All the cultures were standardized with initial OD_{540}=0.05 in 100 mL LB medium. For kinetic analyses, samples were taken at the indicated time points, washed once with PBS (without calcium and magnesium) and bacteria were fixed in PBS-formaldehyde 2% for 15 minutes at room temperature. For resistant subpopulation analysis, samples were collected at an OD_{540}=0.3 before adding polymyxin B 100 µg.ml^{-1} and at an OD_{540}=0.3 after culture treatment by polymyxin B. Samples were then washed once with PBS and bacterial pellets were stored at 4°C until flow cytometry analysis. Thereafter, all samples were analyzed in a FACS Canto II flow cytometer (ROCHE), and tested for GFP quantification and live dead analysis. Ten milliliter of culture were washed once with PBS and resuspended in 1 mL PBS for each color or multicolor staining: Hoechst 33342 strain (ROCHE) which colors dead and live cells (1/10 diluted), Fixable Viability Dye eFluor 660 (ROCHE) (1 µL for 1 mL), Hoechst + eFluor 660 and none treatment. Cells were stained in Hoechst for 15 minutes at room temperature in dark and 30 minutes in e660 in dark and at 4°C. Then the samples were washed once in PBS and fixed as described above.
Forward scatter (FSC), side scatter (SSC) and GFP parameters were set to log, and bi-exponential display was used for the GFP parameter. A total of 30,000 bacteria for each sample were captured unless otherwise indicated and raw data were analyzed with FlowJo version 8.8.6 software (TreeStar). Compensations were done if necessary. Only live cells were count in GFP analysis (Hoechst positive and eFluor 660 negative).

In vivo pathogenicity assays.

The common cutworm, *Spodoptera littoralis*, was reared with a photoperiod of 12 h on an artificial diet at 24°C. Fifth-instar larvae were selected and surface sterilized with 70% (vol/vol) ethanol prior to intrahemocoelic injection. Then, with a Hamilton syringe, groups of 20 larvae were injected with 20 µl a total of 10^3 CFU of bacteria in exponential growth phase culture supplemented with antibiotics when necessary (polymyxin B 100µg.ml^{-1}). Treated

larvae were individually incubated for up to 96 h, and the time at which insects died was recorded. Bacterial concentrations were determined by CFU by plating dilutions onto nutrient agar. Statistical analysis was performed by comparing survival experiments. The rank test (Wilcoxon test) was used to compare mortality patterns as previously described (Jubelin *et al*. 2011).

For bacterial growth kinetics in insect larvae, 60 fifth-instar larvae were injected with 20µL of overnight cultures prior washed once and diluted in PBS (with antibiotics if necessary). 20 and 40 larvae are respectively used to monitor the pathogenicity and the bacterial growth in hemocoel. 4 groups of 2 larvae are surface sterilized with 70% (vol/vol) ethanol, crushed using a TissueLyzer II (Qiagen) at each point of the kinetic in 3 mL LB medium and then centrifuged at 400 *x g* during 2 min to remove large larval debris. Number of bacteria is evaluated by observing an aliquot in fluorescence microscope, the extract is diluted following the estimation and bacterial concentration was determined by CFU plating dilutions onto NBTA nutrient agar supplemented with erythromycin 15 µg.ml^{-1}. To evaluate bacterial concentration of the resistant sub-population the plates were supplemented with erythromycin 15 µg.ml-1 and polymyxin B 100µg/ml.

Results

phoP and *pbgPE* are required to CAMP resistance in *P. luminescens*

The resistance profile of wild type population (TT01), *phoP*, the complemented *phop*/P$_{lac}$phoPQ mutant strain, *pbgE* and the complemented *pbgE*/P$_{lac}$pbgPE mutant strain, toward cationic antimicrobial peptides (CAMPs) was analyzed by minimal inhibitory concentration (MIC). As previously reported (Derzelle *et al*. 2004 ; Bennett et Clarke 2005), TT01 resists to high doses of CAMPs when *phoP* and *pbgE* mutant strains are susceptible at very low doses of antimicrobials especially MIC with polymyxin B (Table 1). The complementated strains (*phop*/P$_{lac}$phoPQ, *pbgE*/P$_{lac}$pbgPE) restore the wild-type resistant phenotypes. The main explanation would be that the wild-type population is completely resistant toward CAMPs and that PhoP and *pbgPE* are fully required to induce CAMPs resistance. However, when a log less CFU of wild-type strain was used as inoculum in the MIC assays, the bacterial growth in presence of high concentration of polymyxin B is weak (Pagès S., data not shown).

PhoP directly controls *pbgPE* and *phoP* expression in *P. luminescens in vitro*

In other Gram negative bacteria, *pbgPE* has been described to be under the indirect regulation of the two component system PhoP-PhoQ, like in *S.* Typhimurium (Gunn et Miller 1996), or under the direct regulation of PhoPQ like in *Y. pseudotuberculosis* (Flamez *et al*. 2007). Moreover, we previously showed that the phosphorylated form of PhoP can directly bind the promoter region of $ail1_{Pl}$ gene, a PagC related protein (Mouammine *et al.* 2014). In order to have a better understanding of PhoP regulon in *Photorhabdus*, we described the interactions between PhoP and *pbgPE* promoter region using electro-mobility shift assays (EMSA), EMSAs were carried out to compare the interaction profiles of different amounts of PhoP protein on the 198-bp *pbgPE* and 206-bp *phoP* promoter regions (figure 1). A recombinant N-terminal His-tag PhoP protein (PhoP-His) was first produced from P_{T7}PhoP-His vector (Table S1). The PhoP-His protein was purified and phosphorylated *in vitro* by incubation with acetyl phosphate. Then, different amounts of phosphorylated and unphosphorylated PhoP-His were mixed with radiolabeled *phoP* and *pbgPE* promoters. A gel shift pattern was observed when 1.5 µM, in the case of *phoP*, and 3.1 µM, in the case of *pbgP*, of phosphorylated PhoP-His was added (figure 1). No shifted bands were observed upon incubation with unphosphorylated PhoP-His. Therefore, PhoP-His protein can specifically bind to the promoter region of *phoP* and *pbgPE* confirming that the active form of PhoP corresponds to the phosphorylated isoform and showing the positive feedback loop of PhoP on its own expression.

Low magnesium activate PhoP-dependent gene expression in *P. luminescens*

It has been shown that low concentrations of Mg^{2+} activate the expression of PhoP-dependent genes in *Salmonella* whereas high Mg^{2+} concentrations repress the system ((for review see (Groisman 2001 ; Kato, Groisman, et Howard Hughes Medical Institute 2008). We also previously demonstrated that in *Photorhabdus* $ail1_{Pl}$ gene, which is a PhoP activated gene, responds to low Mg^{2+} concentrations. To extend our study to *pbgPE* among other genes, we used RT-qPCR approach to evaluate the effect of low Mg^{2+} concentrations on PhoP-dependent genes expression. RT-qPCR was performed on RNA from TT01 strain grown in M9 minimal medium supplemented with 10 µM or 10 mM MgSO4 at an OD around 0.3. The figure 2 shows that in 10 µM MgSO4 condition *pbgP* expression in TT01 is 8-fold more important than at 10 mM whereas it only represent a 2.5-fold increase for *pbgE*. For $ail1_{Pl}$ gene expression is similar to *pbgP* at 10 µM with 9-fold increase. This confirms that *pbgPE* is also induced at low concentrations of MgSO4 in *P. luminescens*. It has previously been shown that in *S.* Typhimurium, suboptimal concentrations of CAMPs can also activate PhoP-

dependent gene expression (Bader *et al*. 2005). The involvement of CAMPs suboptimal concentrations on *pbgPE* expression was investigated and no increasing in *pbgPE* expression was detected compared to wild type strain without treatment (data not shown).

To go further, we defined the transcriptionnal starts of *pbgPE* and $ail1_{Pl}$ genes. The transcriptionnal start of *phoP* was already identified by Derzelle *et al* 2004 (figure 3). RACE PCR approach was used to determine the transcriptional start by analyzing RNA extracted from TT01 cultures in 10 µM MgSO4, the inducing condition. We identified conserved -35 box and more divergent -10 box, two conserved sites for the fixation of RNA polymerase enzyme (figure 3). When compared to *S.* Typhimurium, no conserved PhoP box was identified in *Photorhabdus* and no consensus between the $ail1_{Pl}$, *phoP* and *pbgPE* genes of *Photorhabdus* were found.

Only 0.5 % of the bacterial population resists to CAMPs.

Unlike MIC, antibiogram allows analysis of bacterial resistance towards CAMPs at the individual level. We analyzed the resistance profile of wild type population, *phoP* and *pbgE* mutant and their respective complementation *phoP*/P_{lac}phoPQ, *pbgE*/P_{lac}pbgPE strains (figure 4). Surprisingly, only few colonies from TT01 can grow in the halo containing a gradient of polymyxin B concentration demonstrating that the most part of the wild type strain appears to be susceptible to polymyxin B. In contrast, no clones were observed in the halo for *phoP* and *pbgE* strains. The *phoP*/P_{lac}phoPQ complemented strain has a similar profile than the wild-type strain with more resistant clones (about 7% of resistant bacteria in total population) and the *pbgE*/P_{lac}pbgPE complementated strain has a fully resistance phenotype. We can conclude that the TT01 strain is heterogenous with the major part of its population which is susceptible towards CAMPs and a few part resistant. According to these results, it is likely that the resistant sub-population of *P. luminescens* requires expression of *phoP* and *pbgPE*.

We next quantified the proportion of resistant and susceptible sub-populations in the wild-type strain during bacterial growth by spreading bacteria on nutrient agar plates supplemented or not with polymyxin B (figure 5). We observed that about 0.5% of the wild-type population can resist to CAMPs overtime. When adding polymyxin B in the medium, the percentage of resistant sub-population increases to reach almost 50% in four hours, but if the selection pressure is removed the percentage of resistant bacteria decreases from 50 % to 11% in less than 24 hours and 5% after 38 hours. So the heterogeneity of the wild type population is reversible *in vitro*.

PhoP-dependent genes are over-expressed in the polymyxin-resistant sub-population

The relative expression of PhoP-dependent genes between the resistant sub-population and the WT were analysed. RNA samples were collected from bacteria grown in LB medium (OD = 0.3) and after the polymyxin B treatment when they reach again an OD = 0.3. We observed a *pbgP*, *pbgE* and $ail1_{Pl}$ RNA increases by 4 to 5-fold following the antimicrobial treatment compared to the wild type population (figure 6). Because *pagC* is PhoP-independent in *Photorhabdus* (Mouammine *et al.* 2014) it is used as an internal negative control such as *recA* and *gyr*. We also tested other genes previously described to be involved in resistance towards CAMPs. *galE* and *galU* are two genes implicated respectively in biosynthesis of UDP-galactose and UDP-glucose two precursors of L-aminoarabinose biosynthesis (Easom, Joyce, et Clarke 2010). Addition of L-aminoarabinose confers resistance towards CAMPs when conjugated on LPS lipid A (Bennett et Clarke 2005). Expression of *pagP*, *galE* and *galU* is not statistically dissimilar in resistant bacteria than in WT-population.

To confirm that the emergence of the resistant sub-population is correlated to a higher amount of *pbgPE* expression at the single cell level, we constructed a transcriptionnal fusion between the promoter region of *pbgPE* operon and a destabilized GFP, the TT01/P_{pbgPE}-*gfp*[AAV] reporter strain (figure 7A). Cytometer analysis allowed the quantification of cells expressing *pbgPE* during growth without any CAMPs selection (figure 7B). The maximum of *pbgPE* expressing bacteria was observed during exponential growth phase with almost 15%. Next, the same procedure was used to compare the fluorescence profile of bacterial population during the selection of the resistant sub-population with polymyxin B (figure 7C). After addition of polymyxin B (12 h post treatment), there are 32-fold more live bacterial cells expressing GFP. It is noteworthy that the GFP intensity representative of *pbgPE* expression per cell (X-axis) is not higher in LB than afer polymyxin treatment. Such data are consistent with a selection mechanism of resistant sub-population by a representative of CAMPs, the polymyxin B.

Resistant sub-population is the insect killer.

As the *pbgPE* expressing sub-population allows TT01 growth in presence of CAMPs *in vitro*, we studied the fate of resistant sub-population in insects by performing *in vivo* growth kinetic of TT01 strains (figure 8). At different post-injection time-points, resistant sub-population was quantified by plating extracts of crushed insect larvae (Figure 8A). As previously described (Jubelin *et al*. 2011), after few hours post-injection (hpi), there was a 3-log decrease of the number of alive TT01, this phase is called clearance. Obviously, at this time point (6 to

10 hpi), the alive population reached the number of resistant bacteria (Figure 8B). It is noteworthy that AMPs are synthesized between 4 to 8 hours post infection (Bang *et al*. 2012) which corresponds perfectly to our clearance phase. During the septicemia phase (between 24 and 48 hpi), almost all insects died. It is clear that during bacterial growth resistant sub-population over-competed susceptible population and that septicemia depends on resistant bacteria multiplication. To support that resistant sub-population can kill the insect earlier, we injected pre-selected resistant sub-population with polymyxin B in insects and monitored insect death during time. When injected polymyxin resistant sub-population, the TL_{50} were observed 4 hours earlier compared to TT01 WT population (Figure S1 supplemental data). As observed during *in vitro* cultures, we tested the reversibility of the resistant phenotype after long-term incubation in insects. After 7 day post-injection in the insect cadaver, the wild-type population is back to the proportion observed *in vitro* without any selection, about 0.5% of resistant bacteria in the total population (data not shown).

Discussion

P. luminescens TT01 is a heterogeneous population composed of two sub-populations: one resistant and one susceptible to CAMPs. The resistant sub-population represents 0.5% of the wild type population. Its emergence requires the two-component system PhoP-PhoQ and *pbgPE* genes. We demonstrated that PhoP can activate its own transcription though a positive feedback loop as previously described (Derzelle *et al*. 2004.). Contrary to what have been extensively described in *S.* Typhimurium or in *E. coli*, a direct binding of phospho-PhoP on the promoter region of *pbgPE* operon occurs in *P. luminescens*. The architecture of the phoP regulon is closer of *Y. pseudotuberculosis* where PhoP also directly bind *pbgPE* promoter (Flamez *et al*. 2007). For *S.* Typhimurium the PhoP recognition pattern is a repeated hexameric sequence (G/T)GTTTA-5pb-(G/T)GTTTA (Lejona *et al*. 2003). However this conserved pattern is not found in *Photorhabdus*. Comparison of 5'UTR of the genes directly regulated by PhoP such as *phoP*, *pbgP* and $ail1_{Pl}$ showed no conserved sequence pattern (Figure 3). In contrast, induction of PhoP at low Mg^{2+} concentrations remains conserved between different enterobacteria. There is a debate around Mg^{2+} as an inducer of PhoP-dependent genes. In *S.* Typhimurium Mg^{2+} induces PhoP-dependant genes *in vitro* in culture medium but in macrophages the concentration of Mg^{2+} is from milimolar in concentration and

it has been clearly shown that *phoP* expression was not induced by Mg^{2+} in macrophages even two hours after infection (Martin-Orozco *et al*. 2006). In insects few data are known concerning Mg^{2+} concentrations in insect hemolymph. In fact, it has been suggested that phytophagous insects have high levels of Mg^{2+} in their hemolymph (Whitcomb 2012). So even if Mg^{2+} can promote resistance gene expression *in vitro*, it does not represent a stimulus consistent with the bacterial lifestyle. In *Salmonella* and in *Yersinia,* acidic pH and CAMPs are better candidates for *in vivo* signals inducing PhoP-dependent gene expression. In the different *Yersinia* species, there are variations in resistance genes regulation, indeed PhoP is indirectly involved in *pbgPE* expression (through PmrAB) in *Y. pestis* whereas in *Y. pseudotuberculosis* PhoP directly regulate *pbgPE* expression as in *P. luminescens*. Few are known about *Y. enterocolitica* concerning *pbgPE* expression but this strain is as sensitive towards polymyxin B as the *phoP* mutant of *Y. pestis*. Also a *phoP* mutant of *Y. pestis* is 10-fold more sensitive to killing in macrophages than a *phoP Y. pseudotuberculosis* mutant (Grabenstein *et al*. 2004). Macrophages, like insect hemolymph, contains CAMPs such as defensin or cathelicidin-related antimicrobial peptide (Zaiou et Gallo 2002 ; Selsted *et al*. 1983 ; Lehrer *et al*. 1983). *Photorhabdus* and *Yersinia* have therefore to face the similar host defenses. However *Photorhabdus* and *Y. pseudotuberculosis* have different lifestyles (extracellular and intracellular respectively). A direct link between PhoP and *pbgPE* promoter may be selected to give a better chance for the bacteria to face antimicrobial peptides in these bacteria.

We have also demonstrated that only the resistant sub-population can grow and kill the insect. It is likely that the susceptible bacteria have a *pbgE* behavior that is avirulent in lepidopteran insects ((Bennett et Clarke 2005), Pagès, unpublished data). The *P. luminescens* heterogeneity is clearly essential for bacterial survival and the success of the parasitic life cycle. Somvanshi and colleagues have also found two sub-populations of TT01 in nematodes *H. bacteriophora*, the natural symbiotic partner for *Photorhabdus*. The two sub-populations allow the bacteria to switch between its two hosts the nematode and the insect. The M-form is found in the nematode partner whereas the P-form is present in insect prey (respectively for the mutualistic and pathogenic lifestyle of the bacteria). Here we demonstrate that the P-form of *P. luminescens* TT01 described by Somvanshi *et al* 2012 is, in turn, subdivided in two sub-populations: the resistant and the susceptible one and only the resistant sub-population can grow and develop in insects. Contrary to the M/P forms that allow the bacteria to switch between the two hosts, we can propose a model in which the heterogeneity of the TT01 population permits bacterial survival in various insects. The nematode transmission of *P.*

luminescens to insects is not specific contrary to its mutualistic relationship (Clarke 2014). Indeed, *Photorhabdus* have to kill a wide range of insect larvae mainly in Diptera, and Lepidoptera and less in Coleoptera to endorse a complete life cycle of its nematode hosts (Laumond, Mauléon, et Kermarrec 1979). Each insect family has its own way of defense with different patterns of CAMPs produced. As an example the cecropines are more conserved in Diptera than in Lepidoptera (Hetru, Hoffmann, et Hancock 2002). We therefore propose that the preexisting resistant sub-population is required to successfully invade any insect. This hypothesis is consistent with the reversibility of the resistance observed seven days after insect injection. In addition, the modifications of LPS are pleiotropic allowing the resistance of different classes of antimicrobial peptides while they are cationic. This coping strategy is called a Bet-Hedging strategy and it has been extensively studied in the case of persister cells and sporulation (Lewis 2007 ; Veening, Smits, et Kuipers 2008). The observation of bacterial colonies of *P. luminescens* next to the paper disc in antibiograms (Figure 4) implies that our resistant sub-population is not persister cells. Indeed persister cells enter in dormancy and cannot multiply in presence of antibiotics (Moyed et Broderick 1986). In addition, we also confirmed this result by using ciprofloxacin as previously used in *P. luminescens* (Somvanshi *et al*. 2012) to evaluate the proportion of persiter cells. One log less persisters (0.09%) than resistant cells were obtained (data not shown). As regarding mechanisms regulating phase variation in bacteria, it has previously been shown that bacteria such as *Neiserria spp* and *Helicobacter pylori* can present different populations with various LPS forms. The main underlying mechanism is slipped-strand mispairing (Serino et Virji 2000 ; Wang *et al*. 2000). Slipped strain mispairing occurs when repeated sequences between parental and daughter strain of DNA are misaligned during replication or reparation process resulting in an increasing or decreasing number of repeated sequences (Levinson et Gutman 1987 ; Van Belkum *et al*. 1998). However, the comparison of promoter DNA sequences of *phoP*, *pbgPE* and $ail1_{Pl}$ as well as the *phoP-phoQ* operon from the wild type and the resistant sub-population did not show any mutation, insertion or deletion (data not shown). We also compared the promoter region environment of *pbgPE* and *phoP* genes and no inverted repeated sequenced, responsible for sequence inversion for example, were identified. To reinforce the hypothesis that the switch occurs at the level of *pbgPE* promoter and not at the PhoPQ level, we showed that the PhoPQ-dependent $ail1_{Pl}$ gene transcription occurs in all bacterial cells (Mouammine, unpublished data), that is not observed with *pbgPE* (figure 8C). As a conclusion it is likely that the underlying mechanism responsible for bacterial

494 heterogeneity in TT01 strain is original and probably epigenetic but remains to be identified
495 and characterized.
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Figure legends

Table 1: **TT01 resists at high doses of CAMPs**. Bacterial strains were cultured with increasing concentrations of insect (Cecropin A) and non insect CAMPs (colistin and polymyxin B). All concentrations are indicated in µg/mL

Figure 1: **PhoP directly binds the promoter region of *phoP* and *pbgPE* genes.** Electrophoretic mobility shift assay was carried out to test the binding of PhoP-His protein activated *in vitro* with ACP 10 mM (P-PhoP-His) or non activated PhoP-His (PhoP-His) on *phoP* and *pbgPE* promoter regions. The PhoP-His concentrations indicated are in micromolar. To ensure that the fixation is specific, we used BSA proteins and poly(dI-dC) in the binding buffer.

Figure 2: ***pbgP* and *ail1*$_{Pl}$ expression increased at low concentrations of MgSO4**. Total RNA from TT01 wild-type strain of *Photorhabdus luminescens* was used for RT-qPCR analysis with internal primers specific for the indicated genes. mRNA levels were normalized against those of a reference gene (*recA*). Data are presented as gene expression level for TT01 wild-type strain. The error bars indicate technical replicates. One representative experiment analyzed with ROCHE program.

Figure 3: **No conserved PhoP box identified in PhoP-dependant genes**. Transcriptionnal initiation start evaluated by RACE PCR in TT01 (*ail1*$_{Pl}$ TT01, *pbgP* TT01) were aligned with those already identified in TT01 (*phoP* TT01) and in *Salmonella* (*phoP* salm, *pbgP* salm). In red are identified the -35 boxes and in purple the -10 boxes. The transcriptional start is in green. In the case of *phoP* and *pbgP* from *Salmonella* the identified PhoP box and PmrAB box respectively are underligned

Figure 4: **TT01 wild type strain population is heterogene**. TT01, *phoP* and *pbgE* mutant and their respective complementations *phoP*/P$_{lac}$phoPQ and *pbgE* /P$_{lac}$pbgPE were layed on Agar plates with paper discs at 500 µg and 50 µg of polymyxin B. Antibiogramms mesure the resistance or sensible profil of bacteria against antibiotics. By diffusion in plates polymyxin create a growth inhibitory halo visible except for *pbgE* /P$_{lac}$pbgPE where the strain is completely resistant.

Figure 5: **Only 0.5% of resistant bacteria can resist to polymyxin B in TT01**. By CFU on nutritive agar plates and nutritive agar plates supplemented with polymixin B we assessed the proportion of resistant bacteria during TT01 growth. In the boxes are indicated the percentage of resistant bacteria for each OD tested. The black line represents the growth curve of TT01 strain over time. This experiments were realised at least three times.

Figure 6: ***pbgP, pbgE, phoP*** **and** ***ail*$_{\mathbf{PI}}$** **expression is increased in resistant sub population compared to wild type population**. Total RNA fromTT01 wild-type strain of *Photorhabdus luminescens* cultured with or without polymyxin B was used for RT-qPCR analysis with internal primers specific for the indicated genes. mRNA levels were normalized against those of a reference gene (*recA*). Data are presented as a ratio of values for with polymyxin B condition and without polymyxin B condition. The bars indicate standard errors calculated using Taylor's series. Significant differences (p-value < 0.05) are indicated by asterisks (*). The relative quantification results were obtained from at least three independent experiments with the REST 2009 program.

Figure 7: **32-fold more bacteria express their resistance gene after polymyxin B selection**.
A: Representative scheme of P_{pbgPE}-*gfp*[AAV] the transcriptional fusion between pbgPE promoter and destabilized GFP, the picture represent a GFP-positive bacterium expressing its resistance genes.
B: TT01/ P_{pbgPE}-*gfp*[AAV] was cultured in LB without polymyxin B selection and cytometry course time were assessed. Briefly samples were preleved during each bacterial growth phase, live and dead stained formaldehyde fixed and analyzed in FACS Canto II cytometer. Only live cells are analyzed. Results are presented as dot plots with the Side scatter relative to GFP intensity. Each dot represents one bacterium.
C: TT01/ P_{pbgPE}-*gfp*[AAV] was cultured in LB two samples were collected, one before and one after adding polymyxin B in the culture medium. We use the same representation than in B pannel. All the experiments were performed at least 3 times.

Figure 8: **Resistant sub-population.is the major population during septicemia.**
A : Representative scheme of experimental procedure.
B : After CFU counting of 4 insects larvae crushing by condition at each time point post injection. Black bars represent insect TT01 wild-type strain extracted from insects and spread

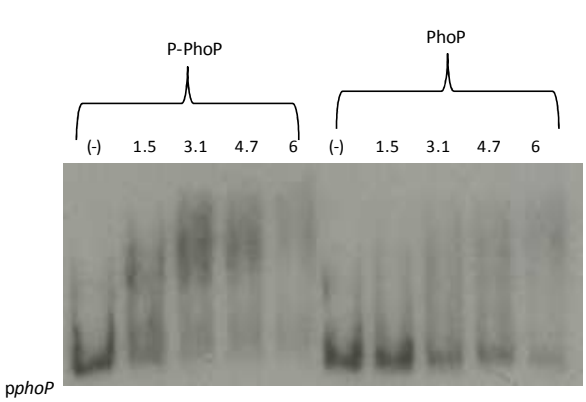
on nutrient agar (black histogramm) or nutrient agar supplemented with polymyxin B (grey histogramm). One representative experiment of more than three.

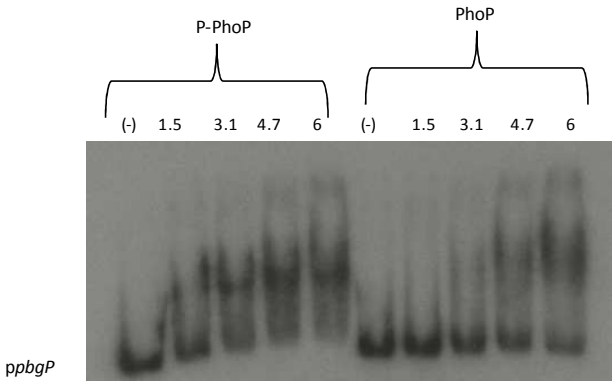
Figure S1: **Resistant sub-population kills quicker insects when compared to wild-type strain**. The results represent the survival curve of TT01 (blue) and pre-selected resistant sub-poplation with polymyxin B (green) over time in insects. More than seven independent experiments were analyzed with R software. Results are statistically different (Wilcoxon Test).

Table 1

Strains	**MIC (µg/mL)**			
	colistin	Cecropin A	Cecropin A (S. frugiperda)	Polymyxin B
TT01	>10.000	>25	> 50	>250
phoP	20	0.8-1.6	6-12	1-3
P_{lac}PhoPQ	>10.000	>25		>250
pbgE	<20	7.8-15.5	6-12	1-2
P_{lac}pbgPE	ND	ND		>250

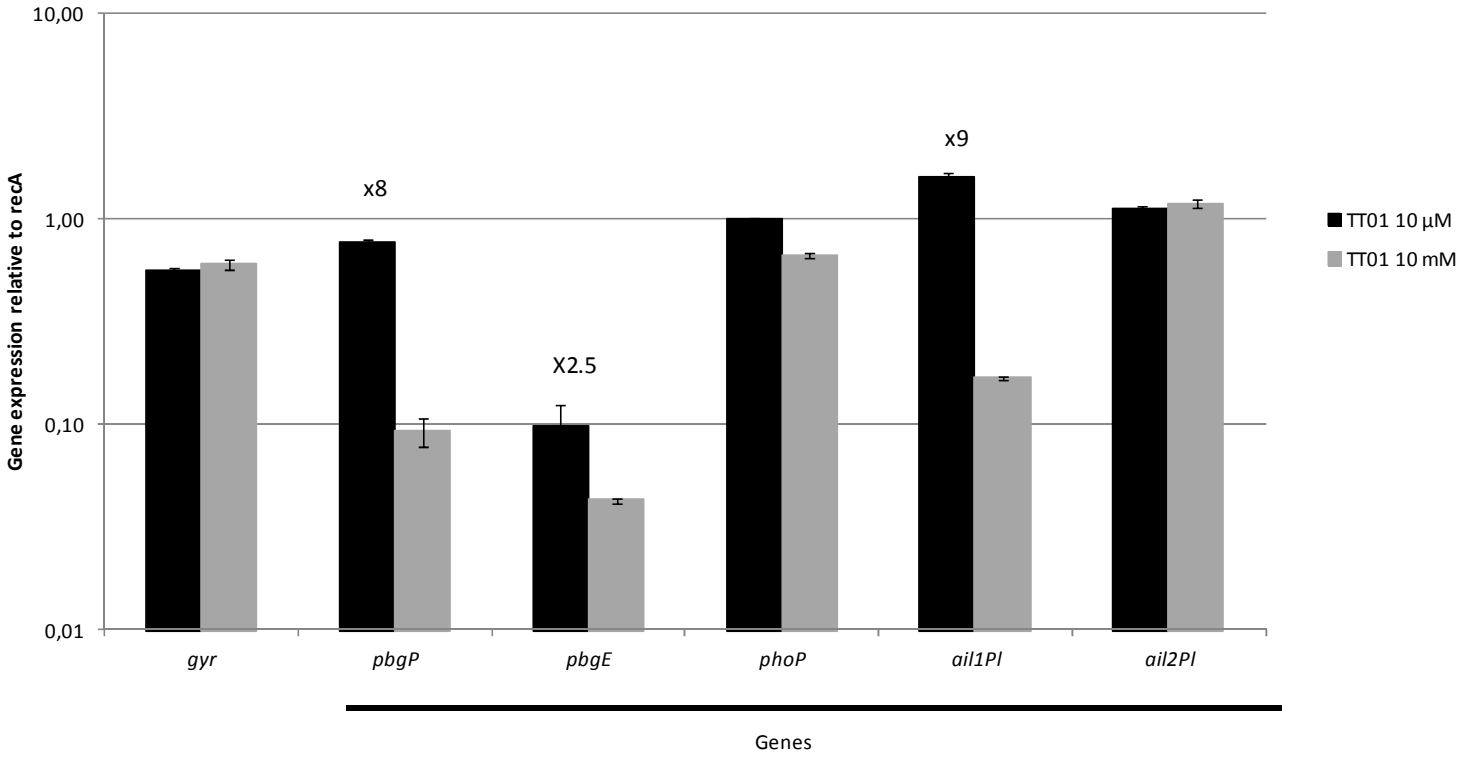
Figure 1





Article 2

Figure 2



Article 2

Figure 3


ail1 TT01 ACAATAAACGAGATTGAGTTGTTTTCTATTGATTGTTGATAAATAATCGCGCCT
phoP TT01 TATTTCCACTTAACGTCTTGCTGTTACAGATCCATGTATCTAACATACTCAGCA
pbgP TT01 TCATACCTGTGTTTACGATTCGTTCAAATATAGTCATCTAATATATCGTTATAT
phoP salm ACTATTTGTCTGGTTTATTAACTGTTTATCCCCAAAGCACCATAATCAACGCTA
pbgP salm CTTCACCTTAATTTCTTAATGTTAATTTAATCTTCATCCAGTAGGGTTCAGCTA

Figure 4

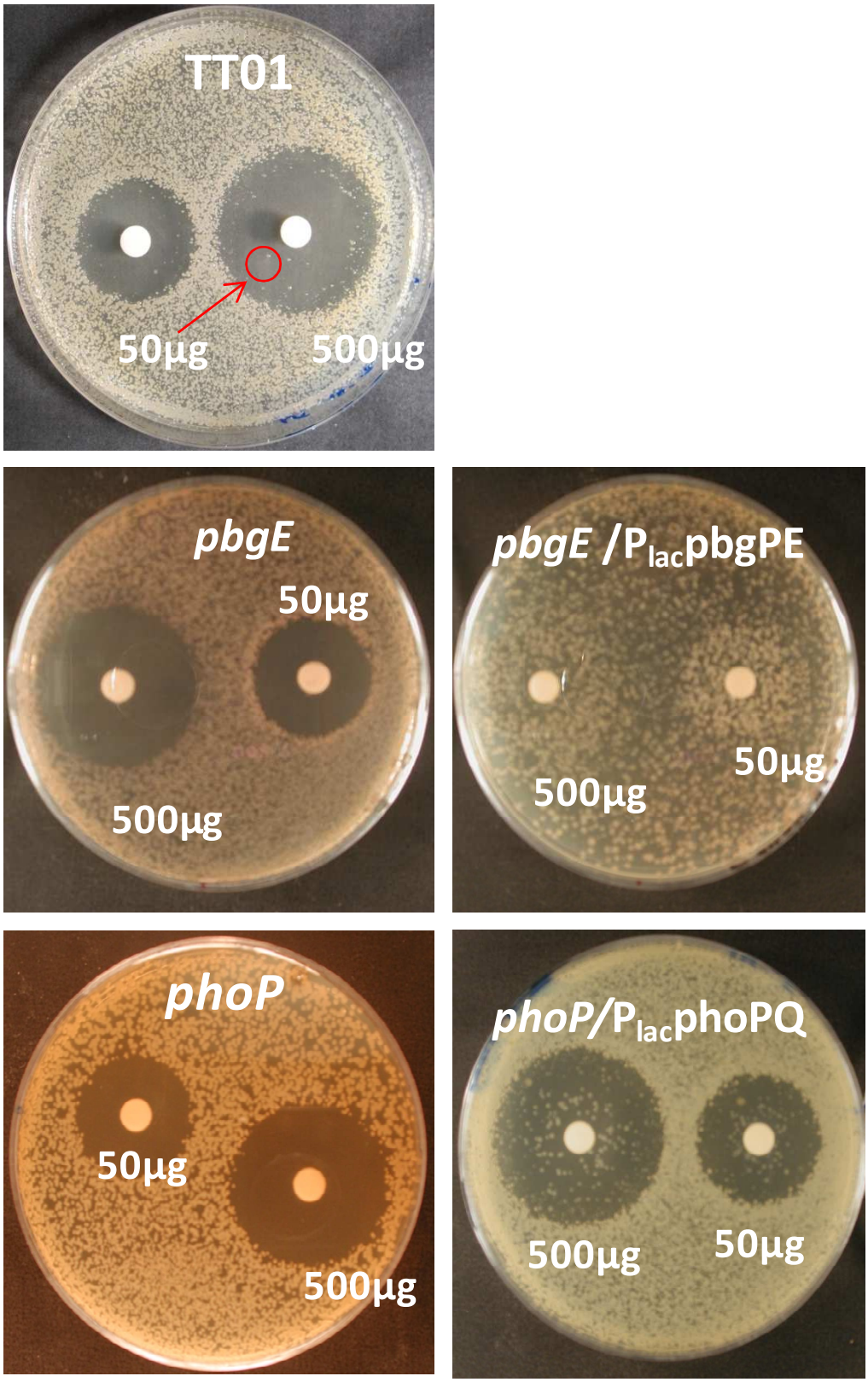

TT01
50µg
500µg
pbgE
50µg
500µg
pbgE /Placpbg PE
500µg
50µg
phoP
50µg
500µg
phoP/PlacphoPQ
500µg
50µg

Figure 5

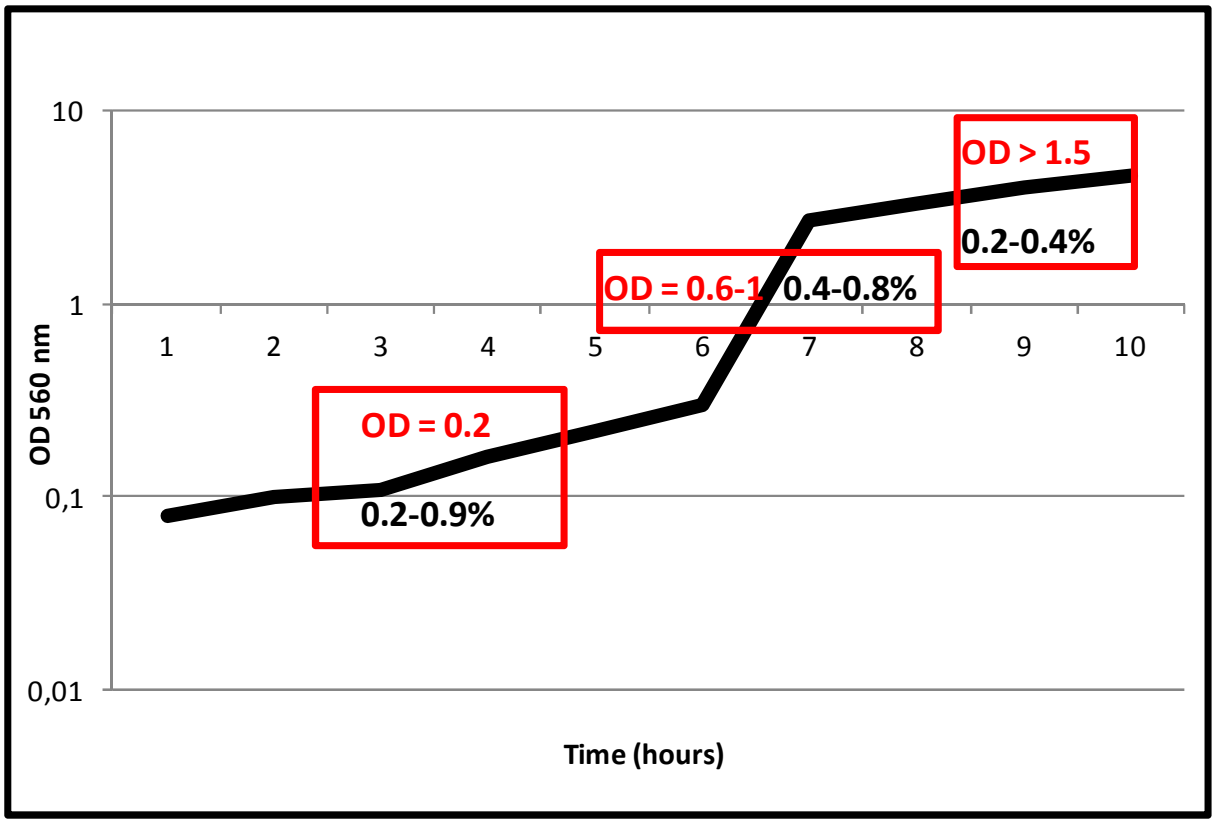

10
1
0,1
0,01
OD 560 nm
1
2
3
4
5
6
7
8
9
10
Time (hours)
OD = 0.2
0.2-0.9%
OD = 0.6-1
0.4-0.8%
OD > 1.5
0.2-0.4%

Figure 6

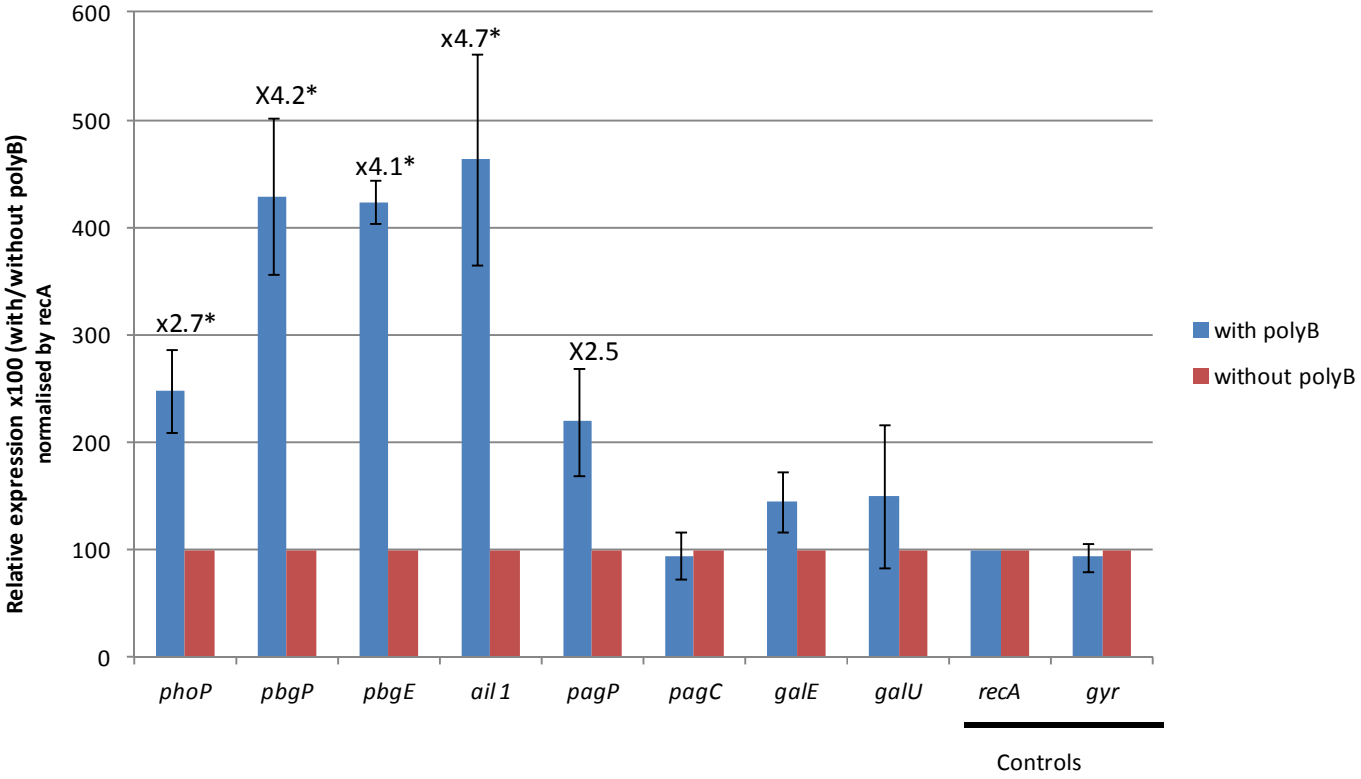
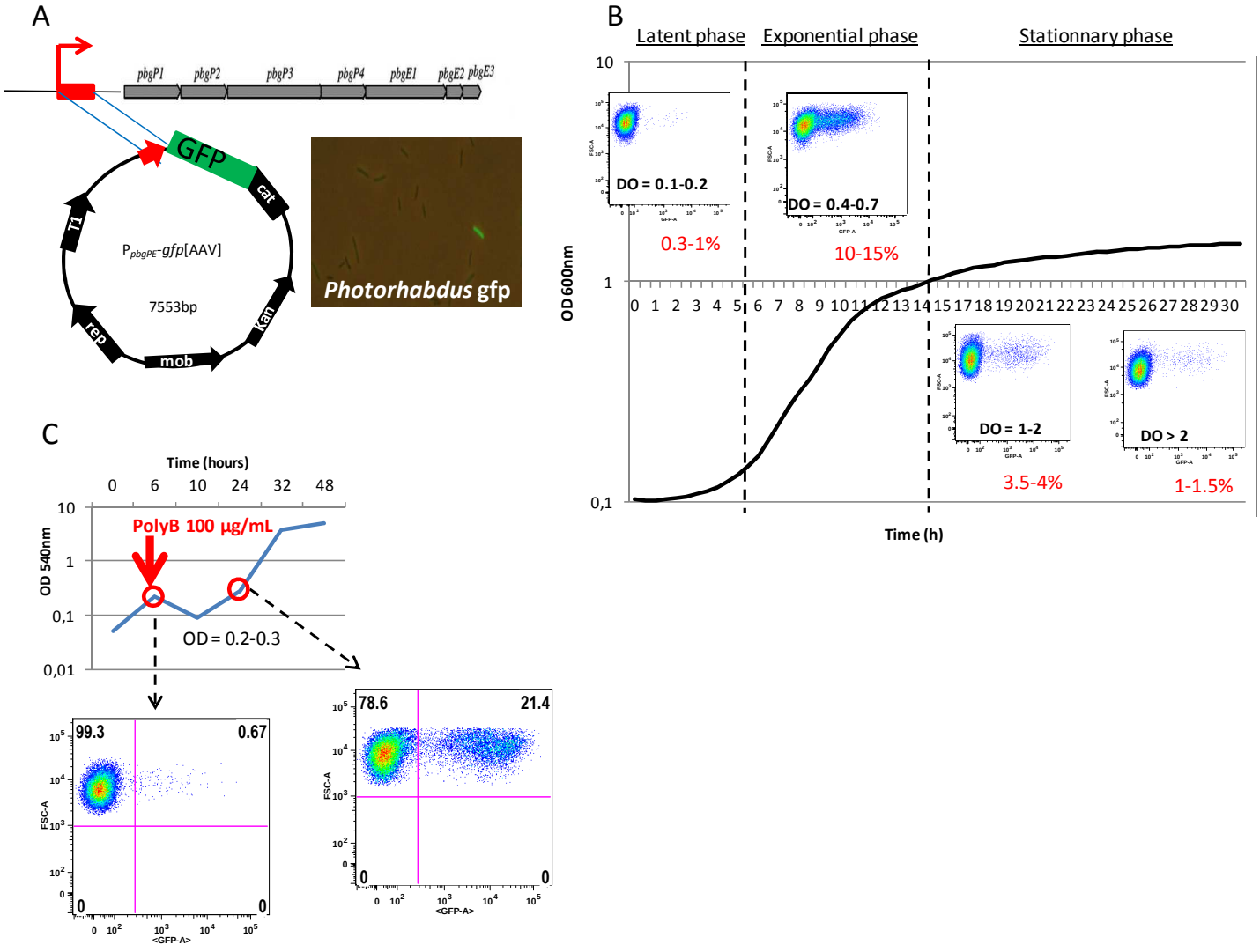

Relative expression x100 (with/without polyB) normalised by recA
600
500
400
300
200
100
0
x2.7*
X4.2*
x4.1*
x4.7*
X2.5
phoP
pbgP
pbgE
ail 1
pagP
pagC
galE
galU
recA
gyr
Controls
with polyB
without polyB

Figure 7



Article 2

Figure 8

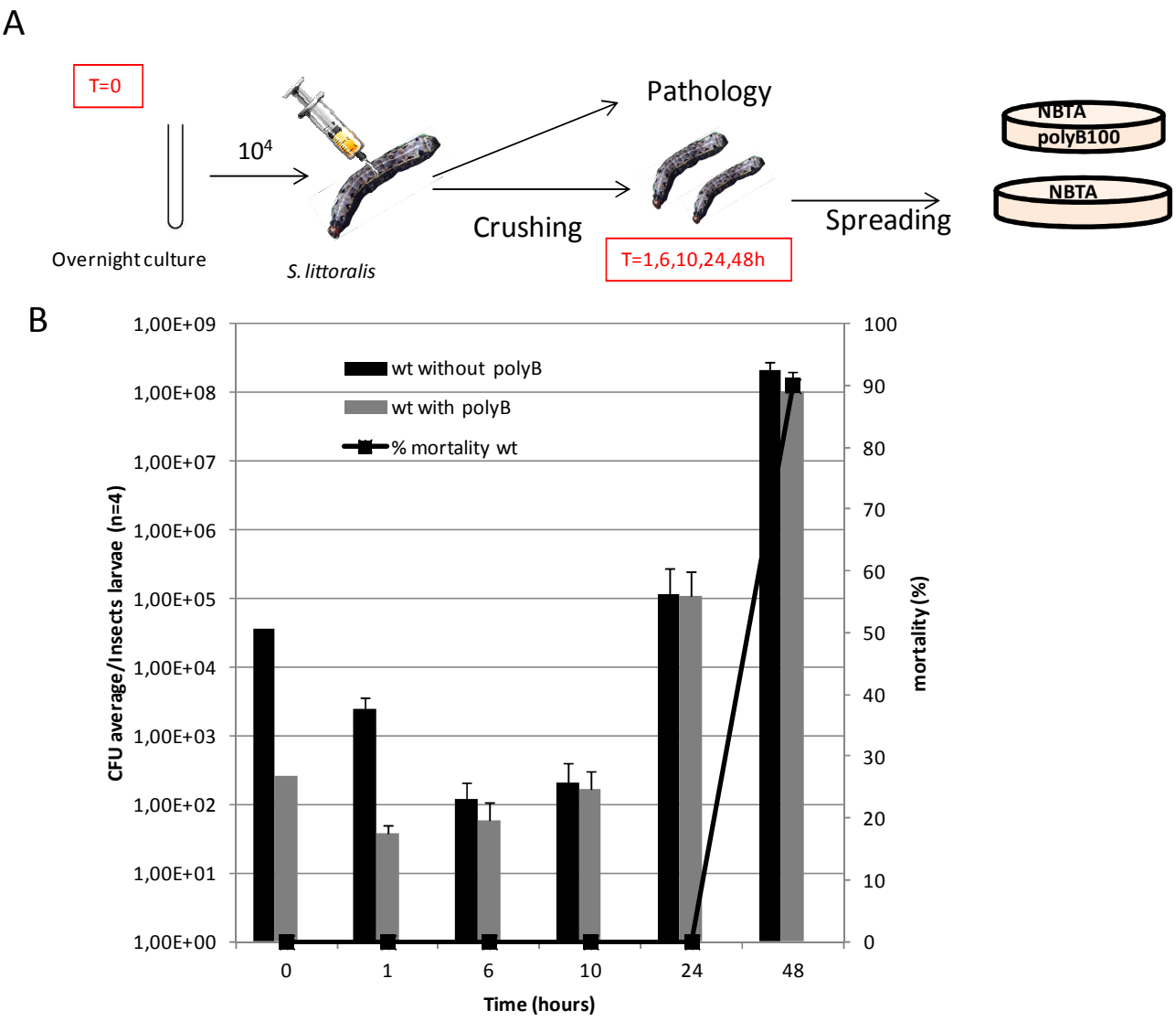

A
T=0
10^4
Overnight culture
S. littoralis
Pathology
Crushing
T=1,6,10,24,48h
Spreading
NBTA
polyB100
NBTA
B
wt without polyB
wt with polyB
% mortality wt
CFU average/Insects larvae (n=4)
mortality (%)
Time (hours)
1,00E+09
1,00E+08
1,00E+07
1,00E+06
1,00E+05
1,00E+04
1,00E+03
1,00E+02
1,00E+01
1,00E+00
100
90
80
70
60
50
40
30
20
10
0
0
1
6
10
24
48

Figure S1

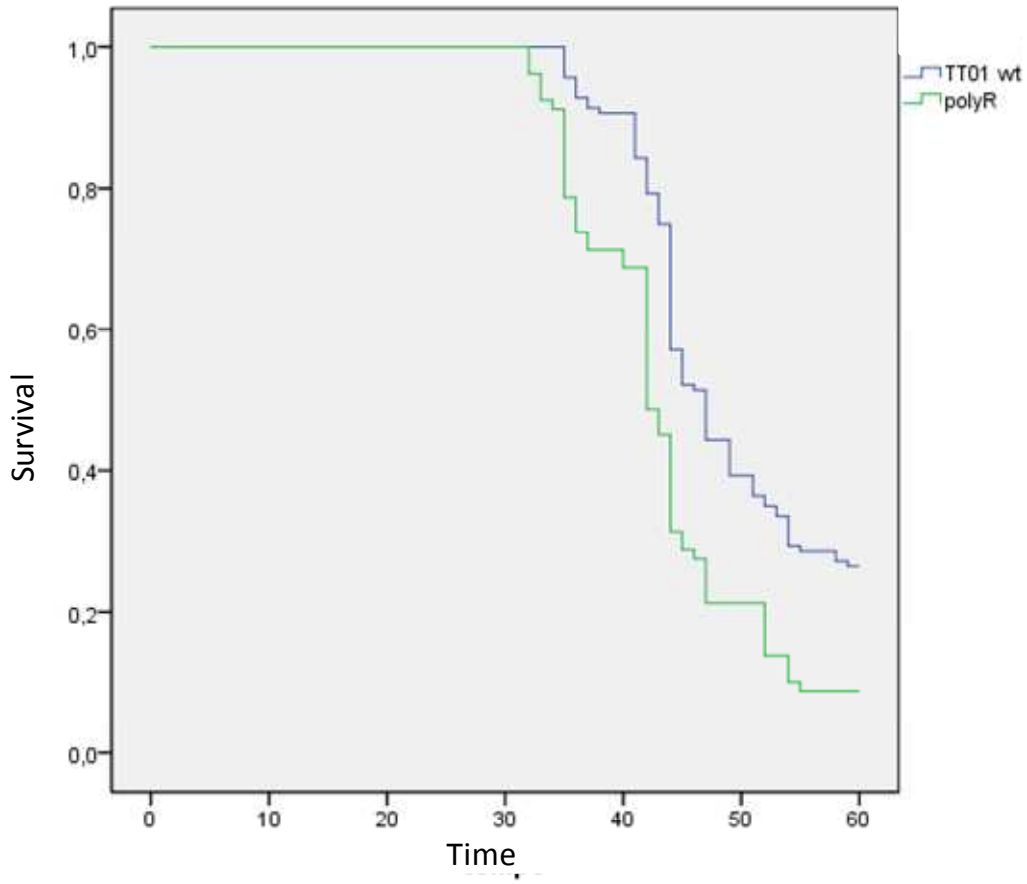


Table S1: Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
***P. luminescens* strains**		
TT01	Strain isolated from the nematode *Heterorhabditis bacteriophora* THO1 in Trinidad; wild-type form	Laboratory collection
phoP	TT01 *phoP*::cat ; *phoP* mutant	(Derzelle et al. 2004)
pbgE	TT01 *pbgE1:*:Kan ; *pbgE* mutant	(Bennett et Clarke 2005)
TT01/ P_{pbgPE}-*gfp*[AAV]	TT01 carrying P_{pbgPE}-*gfp*[AAV] plasmid, Km^R	This study
TT01/ P_{lac}phoPQ	TT01 carrying P_{lac}phoPQ plasmid, Km^R	(Abi Khattar 2009)
TT01/ P_{lac}pbgPE	TT01 carrying P_{lac}pbgPE plasmid, Km^R	(Abi Khattar 2009)
***E. coli* strains**		
XL1 blue MRF'	Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1* gyrA96 relA1 lac [*F'* proAB $lacI^qZ\Delta$M15 *Tn*10 *(*Tet^r*)]*	Stratagene
WM3064	*thrB*1004 *pro thi rpsL hsdS lacZ*ΔM15 RP4-1360 Δ(*araBAD*)567 Δ*dapA*1341::[erm pir (wt)]	(Paulick et al. 2009)
BL21 (DE3) pLysS	F^- *dcm ompT hsdS*($r_B^-m_B^-$) *gal* λ(DE3) [pLysS Cam^R]	Laboratory stock
Plasmids		
pBBR1-MCS5	Broad host range vector, Gm^R *mob*	(Kovach et al. 1995)
P_{lac}phoPQ	2.5-kb fragment cloned into pBBR1-MCS5/*XbaI-PstI* (under P_{lac} promoter control)	this study
P_{lac}pbgPE	7.5-kb fragment cloned into pBBR1-MCS5/*PstI-SacI* (under P_{lac} promoter control)	this study
pPROBE-*gfp*[AAV]	Plasmid (pBBR1 replicon) containing *gfp[*AAV] gene downstream from a multiple cloning site, Kan^R	(Miller, Leveau, et Lindow 2000)
P_{pbgPE}-*gfp*[AAV]	pPROBE with *gfp*[AAV] under the control of *pbgPE* promoter, Kan^R	this study
pETPhos	pET28 replicon, Amp^R	(Canova, Kremer, et Molle 2008)
P_{T7}PhoP-His	pET28 with *phoP*(His-tag) in N-term under the control of T7 promoter; Amp^R	(Mouammine 2014)

Table S2: List of primers used in this study

Primer name	**Primer sequence (5' to 3')**	**Use**
L-PhoPHis-*Nde*I	CGCGCGCCGCATATGCGGATATTGATCGTTGAAGACAACATGTTACTGC	cloning of PhoP-His from *Photorhabdus luminescens* TT01
L-PhoPHis-*Bam*HI	GCGCGGATCCTTACACATCGAAGCGATAGCCTTGGCCCCG	
L-ail1$_{Pl}$-*Eco*	CGGAATTCAAAGCGGGTATCCAGGTTTA	cloning of *ail1*$_{Pl}$ promoter from *Photorhabdus luminescens* TT01
R-ail1$_{Pl}$-*Bam*	CGGGATCCCCTACCGCTACCACTGAAGC	
L-P$_{pbgPE}$-*Xba*I	GCTCTAGATTACAGTCCAGGCTTATGTATGTGCC	cloning of *pbgPE* promoter from *Photorhabdus luminescens* TT01
R-P$_{pbgPE}$-*Kpn*I	CAGGTACCCTATGGAAGAAAGCTATCCATAAAACACAGTCC	
L-P$_{phoP}$-*Xba*I	GCGCTCTAGAAAACATCCGTCTGTTGCTATCC	cloning of *phoP* promoter from *Photorhabdus luminescens* TT01
R-P$_{phoP}$-*Kpn*I	GCGCGGTACCCTCTCCAGCAGGCAGTTATTG	
R-ail1Pl-RACE 1	GGCGTCATTAAATTTTGCGTA	Race PCR amplification to find out *ail1*$_{Pl}$ transcriptional start
R-ail1Pl-RACE 2	TGCAGCACCAATCATACCAT	
R-ail1Pl-RACE 3	ATTATCCAACTCGTAGCGGTATTTC	
R-pbgPE-RACE 1	GCGAATATTCATTGATTCCAATTTA	Race PCR amplification to find out *pbgPE* transcriptional start
R-pbgPE-RACE 2	CAAAAGCATCAATACCCAAT	
R-pbgPE-RACE 3	CTGAGCAATTCCACGCAATA	
L-gyrB	ATACACGAAGAAGAAGGTGTTTCAG	qRT-PCR of an internal region within *gyrB* from *Photorhabdus luminescens* TT01
R-gyrB	TACCTGTCTGTTCAGTTTCTCCAAC	
L-ail1Pl	AGAACATTAGTGGCTTCAGTGGTAG	qRT-PCR of an internal region within *ail 1* from *Photorhabdus luminescens* TT01
R-ail1Pl	ATTATCCAACTCGTAGCGGTATTTC	

L-pagP	TGGTAAATATCGTTACGACGAAGAC	qRT-PCR of an internal region within *pagP* from *Photorhabdus luminescens* TT01
R-pagP	AATAACGGTAATGGGAGTGGAATAG	
L-pagC	GTATCTGCGATAACTTTACCTGCTC	qRT-PCR of an internal region within *pagC* from *Photorhabdus luminescens* TT01
R-pagC	CATTATAGCTGTAGCCAAGATGGAC	
L-phoP	ATTACTATCTGGTCGAGAGCGAAC	qRT-PCR of an internal region within *phoP* from *Photorhabdus luminescens* TT01
R-phoP	TGGTAACATAATCATCTGCTCCTG	
L-pbgP	GAATTCGATGTCTAAAGTTTCATGG	qRT-PCR of an internal region within *pbgP* from *Photorhabdus luminescens* TT01
R-pbgP	GCGAATATTCATTGATTCCAATTTA	
L-pbgE	TTGCCACTAGCCTTATCTACATCTC	qRT-PCR of an internal region within *pbgE* from *Photorhabdus luminescens* TT01
R-pbgE	TTAGTCATAAAACCCATACCACAGC	
L-galE	CAACTAACCCTTATGGCACTTCTAA	qRT-PCR of an internal region within *galE* from *Photorhabdus luminescens* TT01
R-galE	GATAAACATTCCAGACGACCAATAG	
L-galU	AGTTATGGTATTGTCGATTGTCAGG	qRT-PCR of an internal region within *galU* from *Photorhabdus luminescens* TT01
R-galU	ACATAGCGATTGCATCAGTAAGTTG	
L-recA	GTTCAATGGACGTTGAAACTATCTC	qRT-PCR of an internal region within *recA* from *Photorhabdus luminescens* TT01
R-recA	ATCAACACCCAACTTCTTAGCATAG	

Article 2

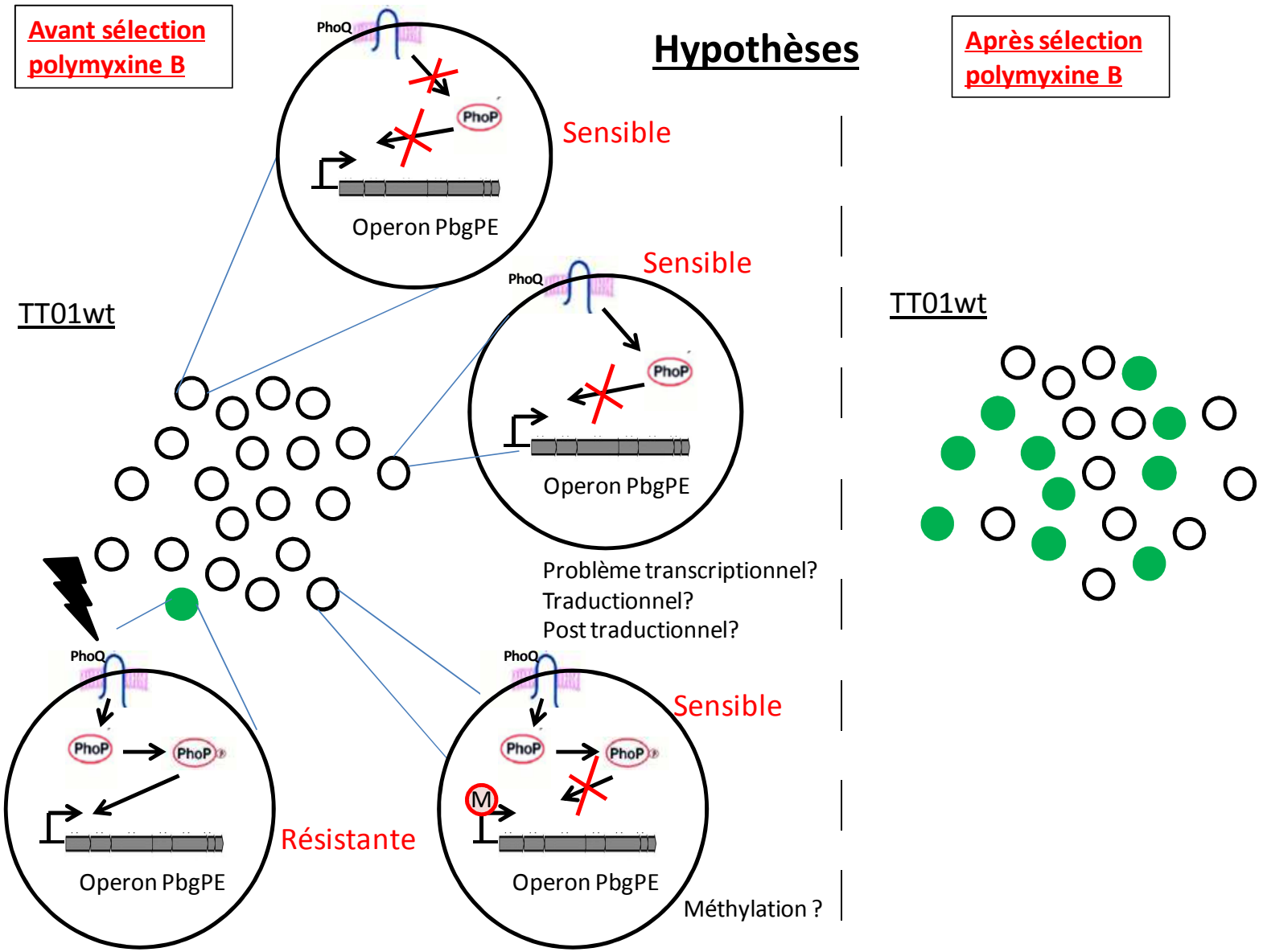


Figure 25 : Schéma des différentes hypothèses pouvant expliquer l'hétérogénéité de la population chez TT01.

IV- Données complémentaires sous forme de résultats -discussions

A- Introduction

Plusieurs mécanismes peuvent être à l'origine de l'hétérogénéité d'une population (cf. Chap I-IV introduction générale) comme des niveaux critiques de transcrits dans une cellule ou de protéine (ex : un faible nombre de transcrits associé à une forte traduction), ainsi que certaines modifications post traductionnelles de la protéine. Enfin, il reste une hypothèse qui est souvent associée à la bistabilité et qui n'a pas encore été testé dans les précédents chapitres : la méthylation.

Dans ce chapitre, nous nous sommes efforcé d'éliminer les hypothèses en partant de ce constat : dans la population sauvage on a une hétérogénéité, dans le mutant *phoP* on perd ce caractère, ainsi PhoP pourrait être la clé du switch. Aussi, nous avons testés si le niveau de transcription ou de traduction de PhoP était à l'origine du switch. En effet nous avons mis en évidence l'existence d'une boucle de rétroaction positive au niveau du promoteur de l'opéron PhoPQ (article 2) qui génère des phénomènes de bimodalité ou de bistabilité (cf Chap I-IV introduction générale). Il peut en être de même avec le niveau de transcription et de traduction de PhoP où il faudrait dépasser une valeur seuil pour déclencher l'apparition de la sous population résistante. De même, une mauvaise efficacité de phosphorylation de la protéine pourrait induire de la bistabilité. Enfin, si le switch n'a pas lieu au niveau de PhoP, c'est qu'il se produit au niveau de de la région promotrice de *pbgPE* Toutes ces hypothèses ont été résumées dans la figure 25.

B- Recherche du mécanisme responsable de l'hétérogénité

L'hypothèse autour de la boucle de rétroaction positive de PhoP

Dans un premier temps, nous avons recherché si l'hétérogénéité de TT01 n'était pas due à un problème de transcription de *phoP* ou de sa faible intensité. Pour cela nous avons dérégulé le système en plaçant les gènes *phoPQ* sous le contrôle du promoteur *lac* (cf. article 2). Cette construction a ensuite été transférée dans les mutants *phoP* afin de regarder si on avait une

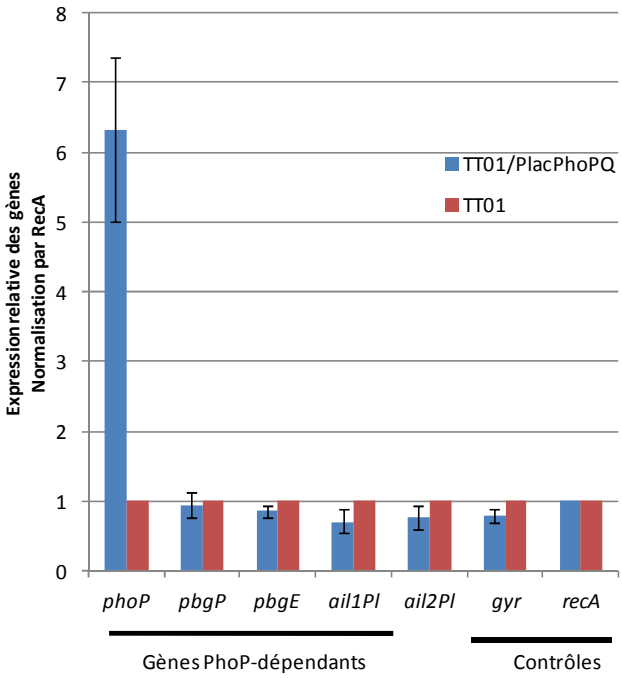


Figure 26 : La transcription de *phoP* n'est pas responsable de l'apparition de la sous population résistante. L'expression des gènes du régulon PhoP entre la souche sauvage et la souche dérégulée pour l'expression de *phoP* est analysée par RT-qPCR. Les écarts type indiquent une erreur standard calculée en utilisant les séries Taylor. Les résultats significatifs (p value <0.05) sont annotés avec un astérisque (*). Les résultats ont été traités par le logiciel REST 2009.

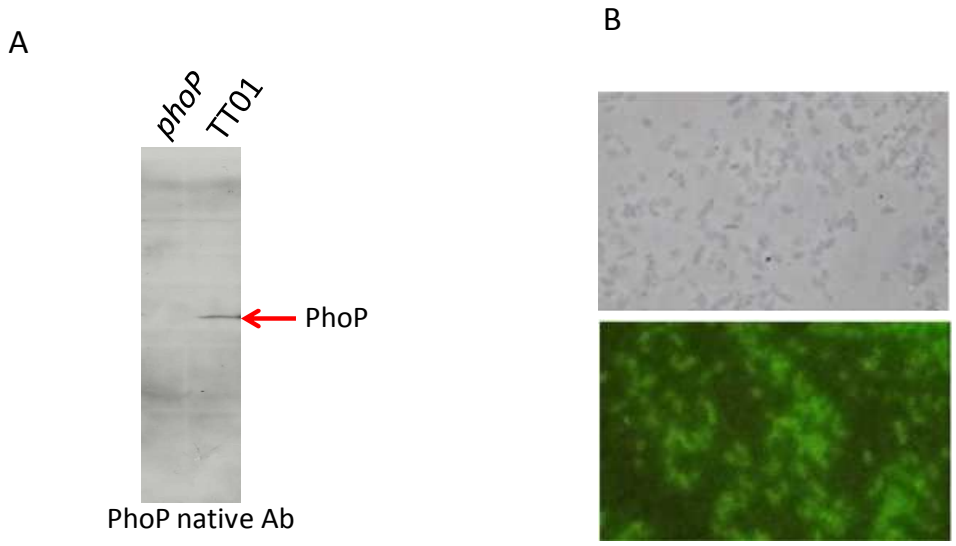


Figure 27. La protéine PhoP est détectée dans toutes les cellules de *P. luminescens* TT01. A : Western blot sur les souches TT01 et *phoP* avec un anticorps spécifique anti PhoP-His. B: Immunofluorescence assay, les cellules TT01 sont fixées sur lame poly-Lysine et perméabilisée pour rendre accessible les protéines intracellulaires à l'anticorps anti PhoP-His. Le signal GFP traduit la présence de la protéine PhoP dans les cellules de TT01.

complémentation de la résistance à 100%. Comme présenté dans l'article 2, les antibiogrammes réalisés sur cette souche ne restaurent pas complètement la résistance à la polymyxine B. En effet, le niveau de bactéries résistantes augmente (contrôlé par CFU) et passe de 0,5% à 6-7% mais on reste loin d'une restauration complète avec 100% de bactéries résistantes. Nous avons contrôlé nos constructions par RT-qPCR afin de s'assurer que les gènes *phoP* étaient bien transcrits à partir du plasmide. Nous avons 6 fois plus de transcrits *phoP* (figure 26), ce qui correspond finalement plus ou moins au nombre de copies du plasmide, pBBR1, alors que l'expression des gènes PhoP–dépendant n'est pas affectée. Nous avons réalisé les mêmes constructions mais cette fois en remplaçant le promoteur *lac* du plasmide par le promoteur naturel de *phoP* chez *Photorhabdus* ou par le promoteur naturel de *phoP* chez *Salmonella*. Ces deux constructions ont ensuite été introduites dans le mutant *phoP*. Là encore, le profil hétérogène des deux constructions était semblable à la souche sauvage. Bien que chez *Salmonella* il ait clairement été montré que PhoP nécessitait une régulation spécifique (« surge » voir (Shin *et al*. 2006)), ces résultats nous ont amené à conclure que la transcription de *phoP* n'était pas à l'origine de l'hétérogénéité de la population sauvage.

L'hypothèse d'une déficience traductionnelle

Puis, nous avons contrôlé la traduction de PhoP dans les cellules. Afin de détecter PhoP, nous avons surproduit et purifié la protéine PhoP marquée histidine (également utilisée pour les EMSA des articles 1 et 2) et un anticorps a été produit. La spécificité des anticorps obtenus à partir des lapins a été contrôlée par western blot puis testée sur les souches TT01 et *phoP* (figure 27). PhoP est bien présent dans la souche sauvage et absent dans le mutant *phoP* (figure 27A). Par immunofluorescence assay, nous avons vérifié si PhoP était traduit dans toutes les cellules bactériennes ou seulement dans 0.5% des cellules (figure 27B). Nous avons pu observer que PhoP était traduit dans 100% des cellules infirmant ainsi un problème de traduction de PhoP qui serait à l'origine de l'hétérogénéité de la population.

L'hypothèse d'une déficience post-traductionnelle

Enfin, la dernière étape a été de tester l'activation de PhoP. A savoir, est-ce que PhoP est capable de fixer le phosphate et est-ce que l'hétérogénéité est due à une activation de PhoP uniquement dans certaines cellules? Nous avons dans un premier temps pu démontrer que PhoP était capable de fixer le phosphate *in vitro*. Pour cela nous avons phosphoryler la protéine PhoP-His purifiée avec de l'ACP *in vitro* puis nous avons fait migrer l'échantillon sur un phosphogel. La particularité du phosphogel est qu'il possède des ions divalents Mn^{2+} ou Zn^{2+} (dans notre cas il s'agit de Mn^{2+}) et un phostag. Le phostag est capable de fixer de façon non spécifiques les phosphates (et donc les formes

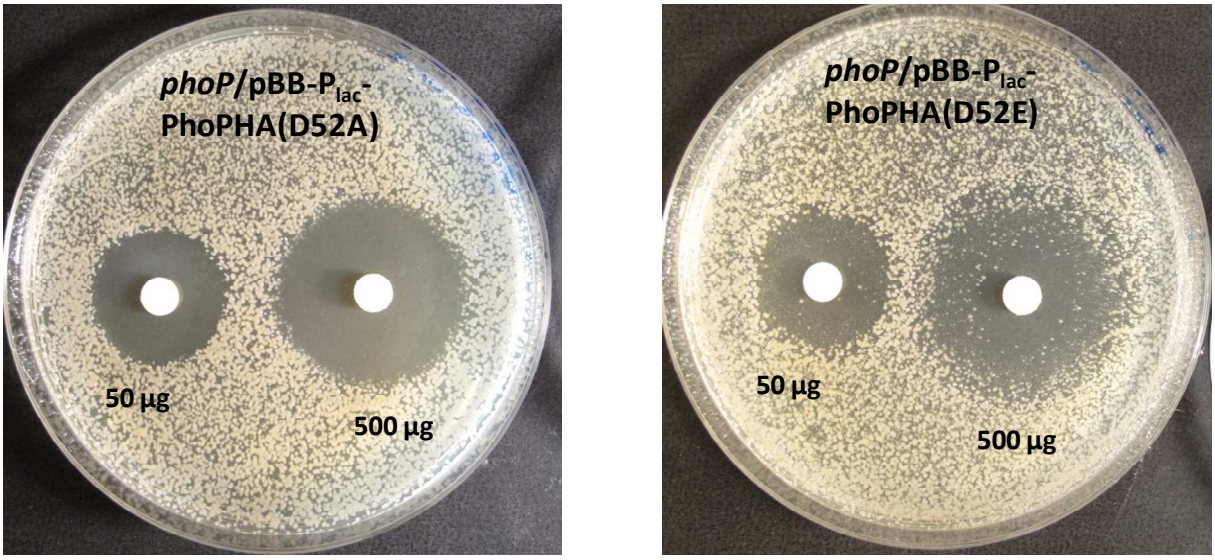


Figure 28 : Rôle de la phosphorylation dans la sous-population résistante. Les souches ont été cultivées en LB et testées par antibiogramme pour leur profil homogène ou hétérogène lorsque PhoP est muté sur son site de phosphorylation. Gauche : *phoP*/pBB-Plac-PhoPHA(D52A) forme non phosphorylable de PhoP. Droite: mutant *phoP*/pBB-Plac-PhoPHA(D52E).

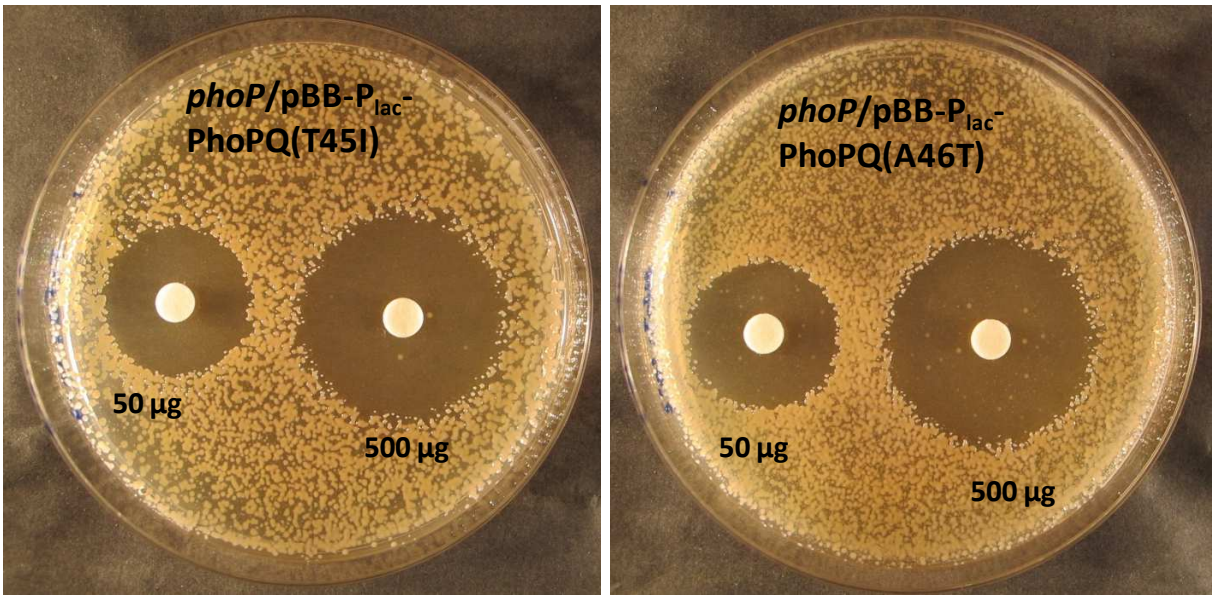


Figure 29 : PhoQ est-il responsable de l'hétérogénéité ?. Les souches ont été cultivées en LB et testées par antibiogramme pour leur profil homogène ou hétérogène lorsque l'on mime l'allèle *pho24* de *Salmonella* Typhimurium. Gauche : mutant *phoP*/pBB-P$_{lac}$-PhoPQ(T45I) modification mimant l'allèle *pho24* (PhoQ constitutif). Droite : *phoP*/pBB-P$_{lac}$-PhoPQ(A46T) mutant mimant le contexte génétique naturel de *Salmonella* Typhimurium.

phosphorylées) des protéines en présence de son co-facteur Mn^{2+}. Cette fixation va entrainer un ralentissement de la forme phosphorylée de la protéine par rapport à la forme non phosphorylée. Nous avons pu démontrer (article 1, figure S1 sup data) qu'en présence d'acétyl phosphate PhoP était présent uniquement sous forme phosphorylée dans sa conformation monomérique et dimérique. PhoP est donc phosphorylable *in vitro*. Nous avons ensuite testé la phosphorylation par l'ACP sur des cultures bactériennes comme précédemment utilisés (Chamnongpol et Groisman 2000). Par CFU sur milieux polymyxine additionné de 50 mM d'ACP, 0,25% de bactéries résistantes ont été dénombrées, soit un résultat comparable à ce qui existe chez la souche sauvage. Les souches TTO1/P_{pbgPE}-*gfp*[mut3] mises en culture en présence d'acétyl phosphate n'ont pas montré une augmentation significative de la fluorescence comparé aux souches témoins ce qui signifie que l'expression des gènes *pbgPE* n'a pas été modifié en présence d'acetyl phosphate (figure 24). Une question reste posée à savoir l'acétyl phosphate est-il dégradé dans le milieu de culture par *Photorhabdus* ou bien n'est-il tout simplement pas métabolisé et internalisé dans les bactéries ? A ce jour nous n'avons toujours pas répondu à cette question, bien que le plus probable soit que l'ACP n'est pas un inducteur et donc que la phosphorylation n'est pas responsable du switch. Cet argument est appuyé par le fait que l'ajout de phosphoramidate (un autre donneur de Phosphate appartenant à la voie de biosynthèse de l'ACP) dans le milieu n'a également aucun effet sur l'hétérogénéité de la population (données non montrées).

L'émergence de la sous-population résistante nécessite la présence du site de phosphorylation sur PhoP

La phosphorylation, bien que non responsable de l'hétérogénéité de la population a bien un rôle essentiel dans l'activation de PhoP. En alignant les protéines PhoP de plusieurs entérobactéries, on peut constater que le site de phosphorylation est conservé (figure 21). Ainsi deux mutants du site de phosphorylation ont été construits. Un plasmide portant le gène *phoP* taggée HA a été muté en remplaçant l'aspartate par une alanine et par un glutamate respectivement (pBB-P_{lac}-PhoPHA(D52A) et pBB-P_{lac}-PhoPHA(D52E)). Ces plasmides ont été ensuite été transféré dans le mutant *phoP* pour voir si les mutations complémentaient ou abolissaient la résistance aux CAMPs. Par antibiogramme, on voit clairement qu'avec le premier mutant *phoP*/pBB-Plac-PhoPHA(D52A) (Figure 28 gauche), la sous-population résistante a disparu et que la souche est complètement sensible aux CAMPs. Ceci montre le rôle essentiel de la phosphorylation et confirme que la forme phosphorylée de PhoP est la forme active ce qui avait précédemment été démontré par les EMSAs (article 1 et 2). Le deuxième mutant consistait à remplacer l'aspartate par un glutamate afin de mimer l'état constitutivement

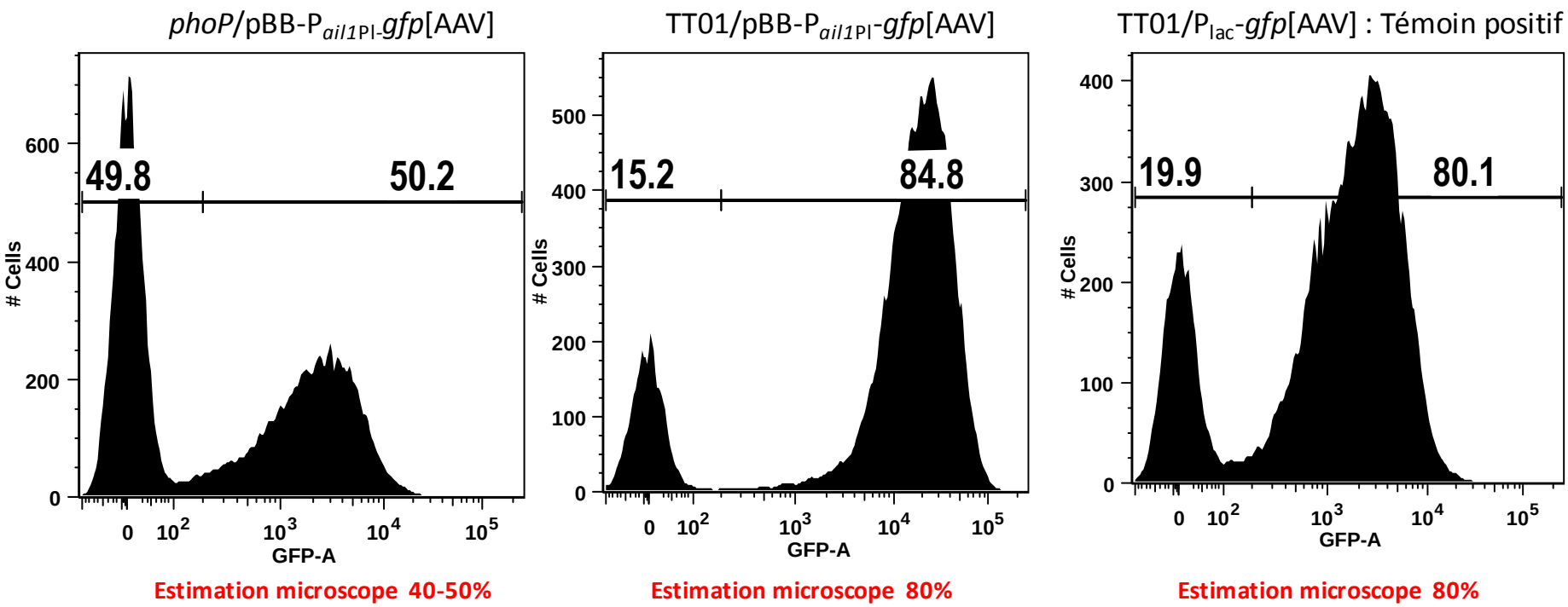


Figure 30 : *ail1*$_{PI}$ est transcrit dans toutes les cellules de TT01. Les constructions pBB-P$_{ail1PI}$-*gfp*[AAV] permettent de suivre l'expression du gène *ail1*$_{PI}$ dans TT01 et *phoP*. Les souches indiquées sur la figure ont été mises en culture jusqu'à une DO de 0,5 environ. Elles ont ensuite été lavées et fixées pour être analysée par cytométrie de flux (Canto II). Les analyses ont été réalisées sur cellules vivantes uniquement. Les résultats sont traités avec le logiciel flowJO et représentent le nombre de bactéries en fonction de l'intensité de fluorescence.

phosphorylé d'une protéine (Molle V, communication personnelle). D'autres mutants constitutifs capable de s'affranchir de l'environnement, ont déjà été construits chez *Salmonella* (Chamnongpol et Groisman 2000). Les antibiogrammes réalisés avec le mutant *phoP*/pBB-P_{lac}-PhoPHA(D52E) montrent une hétérogénéité de la population toujours présente et légèrement supérieure à celle de la souche sauvage (Figure 28 droite). Le nombre de bactéries résistantes a été quantifié par CFU et on a pu montrer qu'environ 7% de la population sauvage était résistante aux CAMPs.

Enfin, une autre possibilité de tester si la phosphorylation était responsable de l'apparition de la sous-population résistante, nous avons construit un plasmide avec uniquement le domaine HTH de *phoP* c'est à dire le domaine de liaison à l'ADN. En s'abstenant du domaine de fixation du phosphate on mime une conformation ouverte de la protéine ce qui reviendrait à une conformation active en absence de phosphorylation. Or nous n'avons pas réussi à surproduire cette construction soit à cause d'un problème de stabilité, le domaine seul n'est pas stable, soit à cause d'une demi-vie extrêmement courte.

En conclusion, toutes ces expériences nous ont permis de conclure que la phosphorylation n'était pas responsable du switch entre les deux sous-populations.

PhoQ responsable de l'hétérogénéité ?

Une autre hypothèse est que les cellules sensibles ne transduisent pas le signal ou ne reconnaisse pas le stimulus. Chez *Salmonella* l'allèle *pho24* ou $PhoP^c$ a été décrit comme présentant la protéine PhoP constitutivement phosphorylée et grâce à une seule substitution dans sa séquence protéique : T48I. En comparant les protéines PhoQ (figure 22), on peut voir qu'excepté *Photorhabdus* toutes les souches comparées (*Salmonella*, *Yersinia* et *Erwinia*) présentent un T alors que seul *Photorhabdus* présente un A. Deux mutants ont été construits: A46T afin de ressembler aux autres protéines PhoQ chez les entérobactéries, T45I afin de mimer le mutant constitutif de *Salmonella*. Comme présenté sur les antibiogrammes (figure 29), le phénotype de résistantes des mutants est identique à la souche sauvage (figure 19) . Aussi PhoQ ne semble pas impliqué dans le switch.

Hypothèse vraisemblable : Un problème en cis dans la région en amont de *pbgPE*

Nous avons pu démontrer que l'apparition du switch n'était due ni à PhoP ni à PhoQ. L'hypothèse la plus probable est que le switch soit lié à *pbgPE*, or la seule connection entre PhoP (qui est essentiel puisque sans PhoP la bactérie devient sensible aux CAMPs et la complémentation du mutant *pbgE* restaure la résistance aux CAMPs avec un profil homogène résistant par antibiogramme) et *pbgPE* est

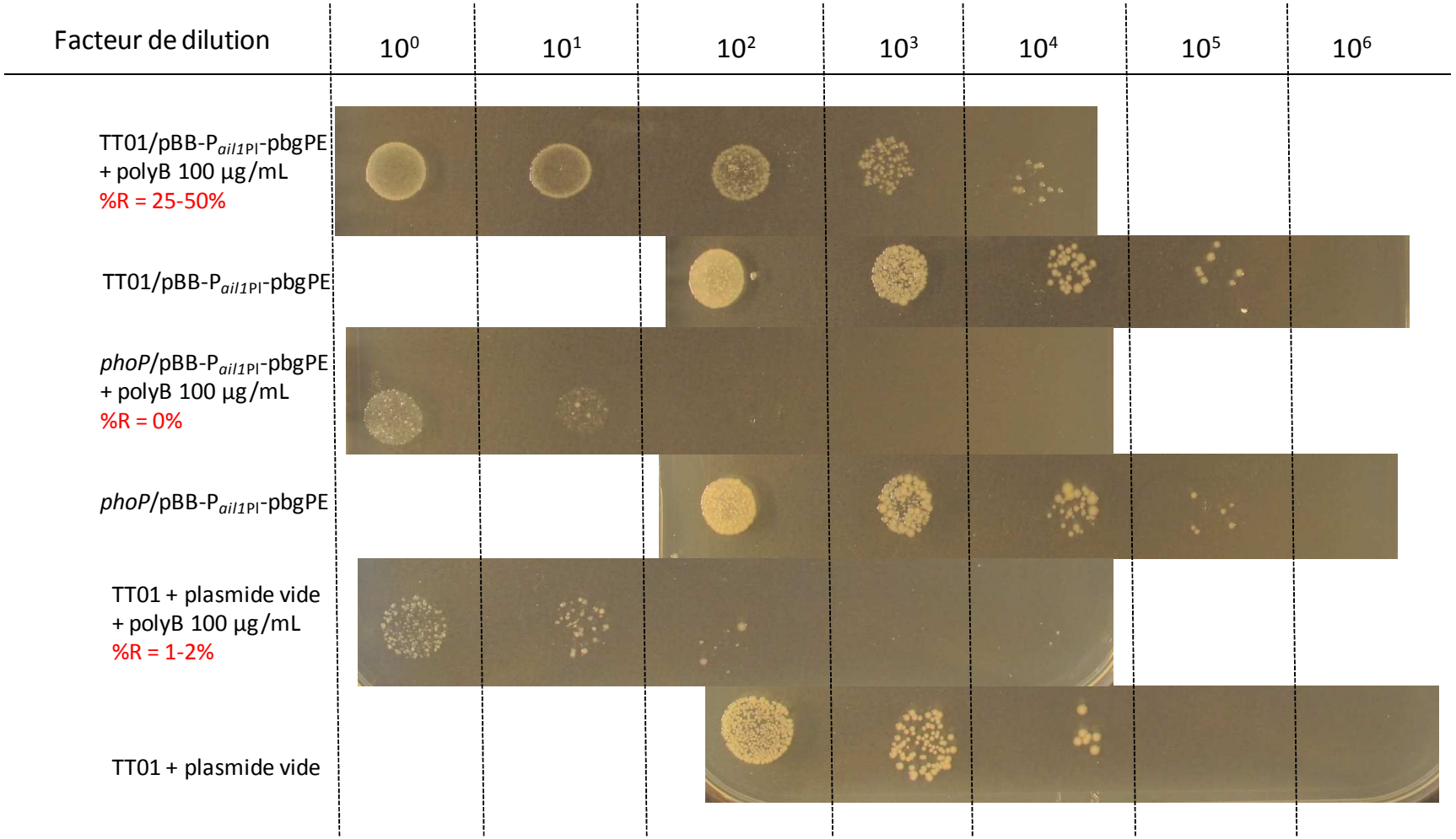


Figure 31 : Spotting des souches TT01et *phoP* complémentée par les constructions pBB-P$_{ail1PI}$-pbgPE en condition avec ou sans polymyxine B. Les souches sont cultivées en LB et à une DO entre 0,6 et 0,8 et diluées. 10 µL de la dilution souhaitée sont déposés sur Gelose nutritive + Gm ou Gelose nutritive + Gm + polymyxine B 100 µg/mL. La dilution permettant la croissance des bactéries est évalué visuellement selon les conditions. %R indique le pourcentage de bactéries résistantes évaluées par CFU.

la région promotrice de *pbgPE*. Afin d'appuyer cette hypothèse, les gènes *pbgPE* ont été placés sous contrôle du promoteur $ail1_{PI}$ qui permet l'expression dans toutes les cellules bactériennes et est activé par PhoP (figure 30 et article 1). Ces analyses montrent que plus de 80% des cellules expriment le gène $ail1_{PI}$ chez TT01.

La figure 31 montre à la fois les résultats CFU et un spotting de ces souches sur milieu avec et sans polymyxine B. Cette dernière expérience, bien que non quantitative, permet néanmoins de visualiser qu'il y a un log de moins de croissance bactérienne pour la souche TT01/ pBB-P_{ail1PI}-pbgPE entre les conditions avec et sans polymyxine B, contre deux log pour le témoin plasmide vide (TT01/pBB-MCS5). Dans le mutant *phoP*, on n'a pas d'activation des gènes de résistance et par CFU on dénombre 0% de bactéries résistantes, en revanche dans la souche sauvage, la présence de la construction a fait passer le nombre de bactéries résistantes de 0,5% chez la sauvage à 25-50% dans la souche TT01/ pBB-P_{ail1PI}-pbgPE. Ceci suggère fortement que la région promotrice de *pbgPE* est à l'origine de l'apparition de la sous-population résistante. Nous aurions donc ciblé la zone responsable du switch mais nous n'avons toujours pas élucidé le mécanisme responsable de l'apparition de la sous population résistante.

L'hypothèse génétique de mutation ou variation de phase

Une altération de la séquence d'ADN peut être à l'origine de l'apparition de résistance chez les bactéries (cf IV- Introduction générale). L'exemple le plus répandu est l'acquisition de mutations qui vont permettre aux bactéries de modifier la cible de l'antibiotique. L'apparition de la sous-population résistante est-elle due à une mutation? Pour répondre à cette question nous avons extrait l'ADN de la souche sauvage TT01 cultivée en LB et en LB supplémenté de polymyxine B afin de ne sélectionner que la sous population résistante. Les ADNs ont ensuite été séquencés et nous avons pu comparer les gènes *phoP*, *phoQ* ainsi que les régions promotrices des opérons *phoPQ*, *pbgPE* et du gène $ail1_{PI}$. Ni les gènes, ni les régions promotrices ne présentent de mutations dans leur séquence entre les conditions avec et sans polymyxine B (résultats non montrés) et les régions du génome de TT01 (Duchaud *et al*. 2003). La sous-population résistante est donc génétiquement identique à la sous-population sensible pour ce qui concerne les gènes contrôlant la résistance aux CAMPs. Le séquençage nous a également permis de rejeter l'hypothèse de la présence d'un glissement de brin ou d'un décalage de trame dû à une insertion ou à une excision d'un ou plusieurs nucléotides car les séquences sont absolument identiques.

La dernière hypothèse a été de vérifier la présence d'une inversion des régions promotrices (variation de phase) des gènes *phoP* et *pbgPE*. En effet, récemment chez *Photorhabdus luminescens,* le « madswitch » correspondant à l'inversion de la région promotrice des gènes *mad* codant pour des

TTACAGT**CCAGG**CTTATGTATGTGCCTGCCGTGAATCTGTACGTATCGTG**GATC**GATAGT
AATTTCCACTTAATTTGCTAAATCATACCTGTGTTTACGATTCGTTCAAATATAGTCATC
TAATATATCGTTATATTATGAATGAATTGGACTGTGTTTTATGGATAGCTTTCTTCCAT

Figure 32 : Identification des sites de méthylation sur la région promotrice de *pbgPE* (198 pb) utilisée dans les constructions P_{pbgPE}-*gfp*[AAV] et P_{pbgPE}-*gfp*[mut3]. GATC : site pour les Dam méthylases. CCAGG site pour les Dcm méthylases.

fimbriae, permet l'expression (état ON) ou la répression (état OFF) des gènes *mad* (Somvanshi *et al*. 2010). Ce madswitch est à l'origine de l'hétérogénéité de la souche sauvage chez le nématode (Somvanshi *et al*. 2012). Grâce à la plateforme d'analyse MAGE (Microbial genome annotation and analysis Platform, https://www.genoscope.cns.fr/agc/microscope/home/index.php) qui recense les génomes de nombreuses bactéries dont *Photorhabdus* et à des outils bioinformatiques spécifiques (http://molbiol-tools.ca/Repeats_secondary_structure_Tm.htm), nous pouvons vérifier la présence ou non de petites séquences inversées-répétées dans le génome à proximité des gènes d'intérêt. Aucune séquence inversée-répétée n'a été mise en évidence à proximité des régions promotrices de gènes *phoP* et *pbgPE*. De même aucune structure secondaire n'a été identifiée dans la région promotrice de *pbgPE*.

L'hypothèse de la méthylation du promoteur *pbgPE*

Un des mécanismes expliquant l'hétérogénéité au sein d'une population est la méthylation. Cela a notamment été démontré chez *E. coli* pour la régulation de l'opéron *pap* (Hernday, Braaten, et Low 2004). Chez *Photorhabdus,* aucune étude n'a montré un quelconque rôle des méthylations chez la bactérie que ce soit pour sa vie symbiotique avec le nématode ou son interaction pathogène avec l'insecte. Chez les bactéries, ce sont principalement les adénines qui sont méthylés (Fang *et al*. 2012). En revanche chez les eucaryotes ce sont principalement les méthylations sur les cytosines qui ont un rôle notamment dans les cancers (pour revue voir (Shaknovich, De, et Michor 2014)). Les principales méthylases des adénines et des cytosines sont respectivement les Dam méthylases (sites GATC) et les Dcm méthylases (sites CCAGG ou CCTGG). Chez *E. coli* ce sont près de 94% des sites GATC qui sont méthylés alors que seuls 0,34% des cytosines des sites reconnus par Dcm sont retrouvées méthylées (Fang *et al*. 2012). Nous avons donc testé si ces deux types de méthylation avaient un rôle dans l'apparition de la sous-population résistante.

En amont de l'opéron *pbgPE*, on trouve les deux sites à savoir GATC et CCTGG (figure 32). Nous avons donc transféré le plasmide P_{pbgPE}-*gfp*[AAV] dans des souches *E. coli* dam+/dcm+ (Xl1 blue) et dam-/dcm- (JM110) afin de tester de façon hétérologue si les Dam et Dcm méthylases avaient un effet sur l'expression du promoteur de l'opéron *pbgPE* (figure 33). La figure 33A montre que lorsque les bactéries sont cultivées en LB le niveau de fluorescence des souches dam-/dcm- est plus de 8 fois supérieur à celui obtenu dans les souches d'*E. coli* dam+/dcm+. En revanche lorsque ces souches sont cultivées en milieu minimum M9 supplémenté de MgSO4 à 10 µM (figure 33B) la différence de fluorescence n'est plus observable et les souches dam+/dcm+ atteignent le niveau de fluorescence des doubles mutantes, ce qui pourrait signifier que l'activation de PhoP permettrait de passer outre la méthylation. Ces expériences ont également été réalisées dans les simples mutants d'*E. coli* dam-