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Substrate independent luminescent phage-based biosensor to specifically detect enteric bacteria such as *E. coli*

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Running Head: Luminescent Phagosensor prototypes, conception and application.

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Abstract

Water quality is a major safety consideration in environments that are impacted by human activity. The key challenge of the COMBITOX project is to develop a unique instrument that can accommodate several biodetector systems (see the accompanying COMBITOX papers) able to detect different pollutants such as bacteria, toxins and heavy metals. The output signal chosen by our consortium is based on luminescence detection. Our group recently developed phage-based biosensors using *gfp* as a reporter gene to detect enteric bacteria in complex environments such as sea water, and the main challenge we faced was to adapt our biodetector to a luminescent signal that could fit the COMBITOX project requirements. Another key point was to use a substrate independent reporter system in order to avoid substrate addition in the detection prototype. This paper describes the development of a phage-based biodetector using a luminescent and substrate independent output to detect some enteric bacteria, such as *E. coli*, in water samples. We have successfully engineered various prototypes using the HK620 and HK97 bacteriophages that use different packaging systems and both proved functional for the integration of the full *luxCDABE* operon controlled by 2 different bacterial promoters. We show that the *luxCDABE* operon controlled by the *PrpIU* bacterial promoter is the most efficient in terms of signal emission. The emission of luminescence is specific, and allows the detection of 10^4 bacteria per ml in 1.5 hours post infection with no concentration nor enrichment step.

keywords: biodetector, temperate phage, water quality, luminescence

I. Introduction

1
2 Drinking and bathing waters are important vectors of infectious diseases since they constitute important
3 reservoirs for pathogens that belong to different taxa such as viruses, bacteria, eukaryotic parasites and
4 fungi. Pathogens present in waters are able to infect naturally present organisms but also humans,
5 terrestrial and aquatic animals. The variety of bacterial pathogens that affect mariculture systems is
6 important and causes tremendous economic costs (Toranzo et al., 2005). However, the main concern has to
7 do with human health since many human pathogens are found in waters and may cause infectious diseases
8 through ingestion, inhalation or just contact (Pond and World Health Organization, 2005). The oral route is
9 the main way to infections and water quality monitoring is the best way to avoid them. In this case, a vast
10 majority of the bacteria responsible for water driven infectious diseases are enteric bacteria such as
11 *Campylobacter*, *Escherichia coli*, *Salmonella*, *Shigella*, *Yersinia* and *Vibrio* species. Nowadays, it is difficult to
12 rapidly identify specific pathogens, therefore water quality is assessed by looking for indicators of fecal
13 contamination such as *E. coli* and gut enterococci. Ideally, a good indicator must fit to the following criteria:
14 (i) it should develop, or at least, survive in the environment, (ii) it should persist in water and be resistant
15 to the same treatments as pathogens, and (iii) it must be absent, or at very low density, in a non-polluted
16 environment and highly enriched in a contaminated one (Stewart et al., 2008). *E. coli* and fecal enterococci
17 are the most frequently used indicators. *E. coli* represents more than 94% of total coliforms found in animal
18 and human feces and is almost absent in other environments (Edberg et al., 2000). Enterococci are able to
19 grow at 44°C, are abundant in feces and survive, without dividing, in the environment (Byappanahalli et al.,
20 2012).

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36 Since their discovery (D'Herelle, 2007; Twort, 1915), bacteriophages, the viruses that specifically infect
37 bacteria, have been used as biotechnology and molecular biology tools and therapeutics (Goodridge and
38 Abedon, 2003; Haq et al., 2012; Schmelcher and Loessner, 2014). Several phage-based methods have been
39 developed to detect bacteria using engineered phages. They use a variety of reporter systems that
40 generate fluorescent, luminescent or colorimetric signals (Derda et al., 2013; Edgar, 2006; Loessner et al.,
41 1996; Mosier-Boss et al., 2003; Piuri et al., 2009). The main advantages of the phage systems is that the
42 detection is highly specific and allows to discriminate between alive and dead cells. Our group recently
43 developed a phage-based system couple to flow cytometry that allows specific and highly sensitive
44 detection of enteric bacteria in water samples (Vinay et al., 2015). In order to fit the COMBITOX project
45 requirements (Ansaldi et al., 2015), we decided to adapt our system to luminescence. Indeed, this project
46 aimed to combine several biodetection systems, including toxins, heavy metals and enteric bacteria, within
47 the same automated station. To achieve this goal, our consortium decided to use luminescence as the
48 common output signal. Our main task in this project was thus to adapt our fluorescent phage-based
49 biosensors to luminescence. Several bioluminescence reporter phage systems have been developed so far.
50 Most of them use the reporter genes *luxA* and *luxB* genes allowing the production of luciferase without the
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1 *luxCDE* genes involved in the production of the luciferase substrate, a fatty aldehyde (Kuhn, 2007; Loessner
2 et al., 1996; Schofield et al., 2009; Waddell and Poppe, 2000). With these biodetectors, the addition of
3 exogenous substrate post-infection is required to detect bacteria. The relatively large size of *luxCDABE*
4 operon (approximately 6 kb) constitute a limitation considering the genome size of a temperate phage (35-
5 60 kb average) that is packaged into the viral capsid. However, a *luxCDABE* reporter phage has been
6 recently developed by replacing nonessential genes of the *Salmonella* temperate phage SPC32H by the
7 entire *lux* operon (Kim et al., 2014). Phage genomes are packaged according two different packaging
8 mechanisms, the *cos* and *pac* systems. In both cases concatemeric phage DNA is used as a substrate for
9 packaging. In *cos* phages, an enzyme, called terminase, recognizes a specific sequence (*cos*) that is cut to
10 form cohesive ends. With this system, the size of the packaged DNA is constant. In *pac* phages, only the
11 first copy of the concatemeric DNA is cut at the *pac* site position, the following molecules are being
12 packaged through a headful mechanism. The headful packaging system is more tolerant, allowing the
13 packaging of up to 110% of the size of the phage genome. In this paper, we describe the strategy we used
14 to develop substrate independent reporter phages using both *pac* and *cos* phages designed to detect
15 different *E. coli* strains. We have constructed and characterized several prototypes using the HK620 and
16 HK97 bacteriophages that use different packaging systems and both proved functional for the integration
17 of the full *luxCDABE* operon under the control of the *PrpIU* bacterial promoter. We show that the emission
18 of luminescence is specific, allowing the detection of 10^4 bacteria per ml in 1.5 hours post infection with no
19 concentration nor enrichment step. Further development will aim to develop phagosensors adapted on
20 demand to the detection of any human or animal pathogen that may be present in surface water.
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II. Results

1. Promoter choice and phage engineering

The challenge of phage engineering was to integrate the entire *lux* operon controlled by a strong bacterial promoter and associated with a kanamycin resistance cassette and homology regions of 80 bp at each extremity to allow homologous recombination. Altogether, the DNA fragment size is nearly 8 kb long (Fig. 1). We made a first construct in phage HK620, which is an *E. coli* TD2158 specific temperate phage belonging to the *Podoviridae* family (Dhillon et al., 1998). It packages DNA through a headful mechanism and thus its genome contains *pac* sites (Clark et al., 2001). We designed two HK620 reporter phages in order to optimize bacterial detection and to obtain a strong luminescent signal. The expression of *luxCDABE* operon was controlled by either *PrpoD* or *PrpIU* promoters, two strong $\sigma 70$ promoters in *E. coli*. The target region for homologous recombination was chosen in between two convergent genes *hkcE-hkcF* to avoid the disruption of any transcriptional unit or promoter region. The same region has been successfully used for *gfp* gene insertion and engineering had no influence on the life cycle of HK620 (Vinay et al., 2015). The construction was done using the λ Red recombineering method (Datsenko and Wanner, 2000). Briefly, PCR fragments containing the entire *luxCDABE* operon under the control of either *PrpoD* or *PrpIU* promoters associated to the divergently oriented *kan^R* cassette and flanked by 80 bp of sequence homology with the target sequences (Fig. 1) were electroporated into *E. coli* LCB6205(HK620) lysogenic strain carrying plasmid pKD46 that allows the production of the λ Red recombinase that promotes efficient homologous recombination in *E. coli* (Datsenko and Wanner, 2000). Thus, the insertion of the full size reporter operon under the control of either *PrpIU* or *PrpoD* promoters in the *hkcE/hkcF* region gives HK620::*PrpoD-luxCDABE* and HK620::*PrpIU-luxCDABE* recombinant phages (Fig. 1 and construction details in the Material and Methods section). Both recombinant phages were efficiently induced with mitomycin C (MMC), the classical inducer of the SOS response, and produced infectious phage particles (data not shown). Moreover, the presence of the large insertion did not affect the production of the engineered phages since they were produced with equivalent titers compared to the WT phages (data not shown). The bacterial growth and culture luminescence upon infection with the 2 recombinant phages were monitored using a microplate reader (Fig. 2). When *E. coli* LCB6205 was infected with the WT phage, the luminescence signal was homogenous and was slightly higher from what observed under non-infected conditions. Compared to the growth curve obtained with non infected bacteria, growth profiles of infected bacteria show a re-growth phase post-lysis characteristic of temperate phages. Indeed, during the first 1.5 hours, a slight bacterial growth was observed, immediately followed by a typical lysis phase. Later on, once lysogeny is established, the lysogens grow exponentially until the stationary phase is reached. However, re-growth started 5 hours post-infection with HK620::*PrpIU-luxCDABE* versus only 2 hours for WT HK620 or HK620::*PrpoD-luxCDABE*. Luminescence intensities measured 1.5 hours post-infection were around 1000-folds higher with HK620::*PrpIU-luxCDABE* than those measured with WT phage or HK620::*PrpoD-luxCDABE*. Together, these

1 results indicate that the engineered phages are able to infect their host, which in turn produced
 2 luminescence. The growth delay observed with HK620::*PrpIU-luxCDABE* might be a consequence of the
 3 promoter strength. Indeed, *PrpIU* is a stronger promoter than *PrpOD* (Zaslaver et al., 2006), and thus higher
 4 amounts of luciferase and substrate are produced when using *PrpIU* as a promoter. We suspect that their
 5 accumulation may be slightly toxic and interfere with early growth. However, it is striking that the *PrpIU*
 6 construct generates a much higher luminescence intensity compared to the *PrpOD* one at a early time point
 7 post-infection in agreement with the respective promoter strengths. The *PrpIU* construct was thus chosen
 8 for further experiments.

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 11 In order to check if the substrate produced by the cells could be a limitation, we performed a similar
 12 experiment with decanal addition prior measurement. An infection assay was performed and incubated 2
 13 hours at 30°C, luminescent was measured prior and after addition of 5 µL of 52 mM decanal solution in
 14 ethanol 50%. A luminescent signal of 180680 ± 9661 RLU was measured prior decanal addition, whereas
 15 the signal measured in the presence of decanal was 156710 ± 8160 RLU. This result indicate that the
 16 substrate produced in the cells is not a limitation for the technique. A very important result is that we were
 17 able to engineer a 38 kb genome and produce homogenous and viable phage particles by adding a reporter
 18 cassette of 8 kb without having to remove any part of the original phage genome, suggesting that this
 19 phage genome is highly tolerant to size increase.

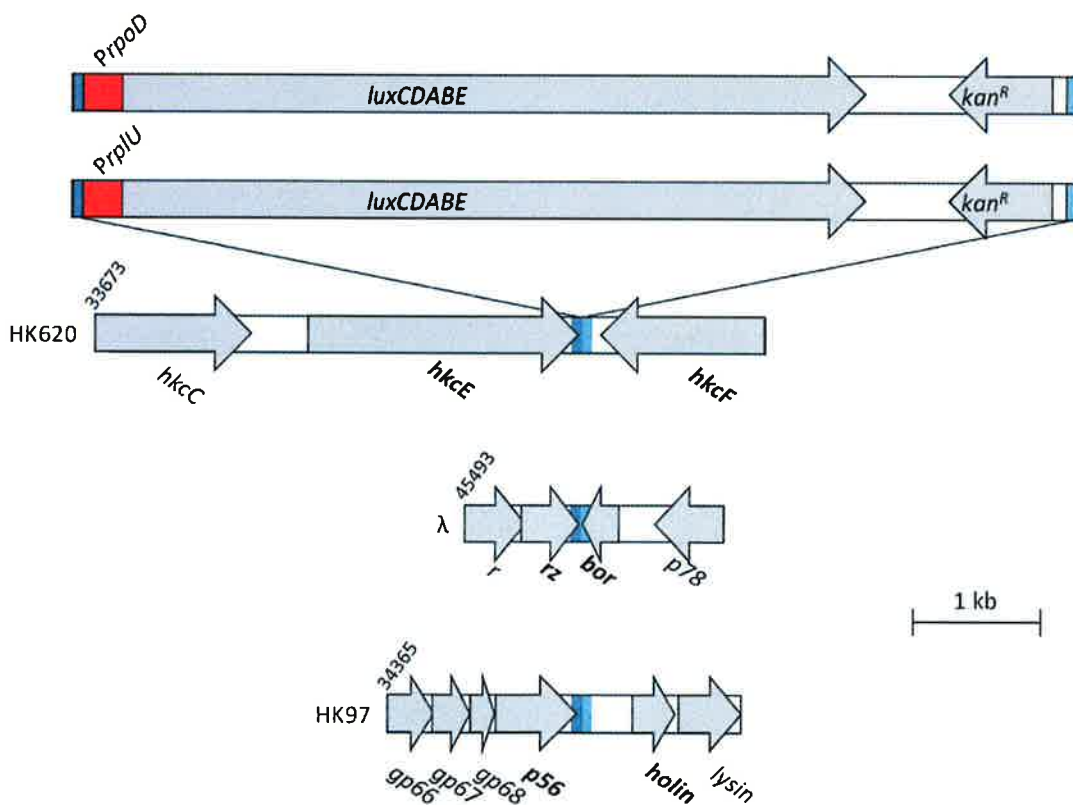


Figure 1. Schematic representation of the strategy to engineer temperate phage genomes. PCR fragments (top) containing the *luxCDABE* operon under the control of either *PrpIU* or *PrpOD* (red) and a kanamycin

resistance cassette (*kan^R*) are flanked by 80 bp homologous (blue) to different intergenic regions in phages HK620 (*hkcE/hkcF*), λ (*rz/bor*) and HK97 (*p56/holin*). Numbers indicate the position on the respective genomes.

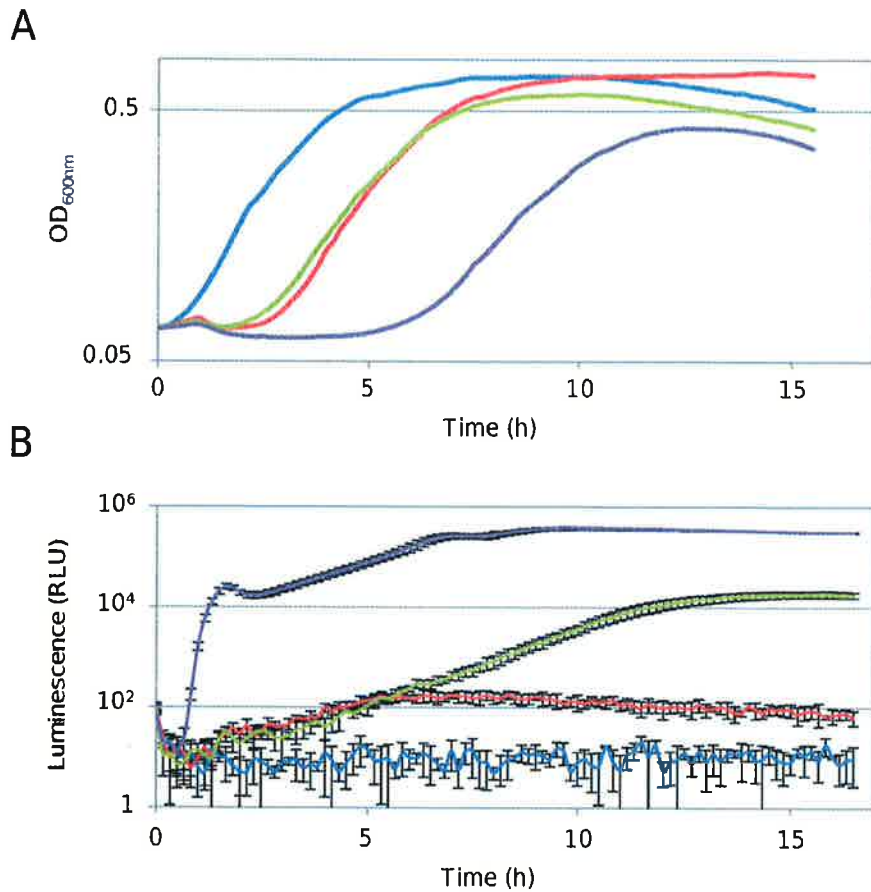
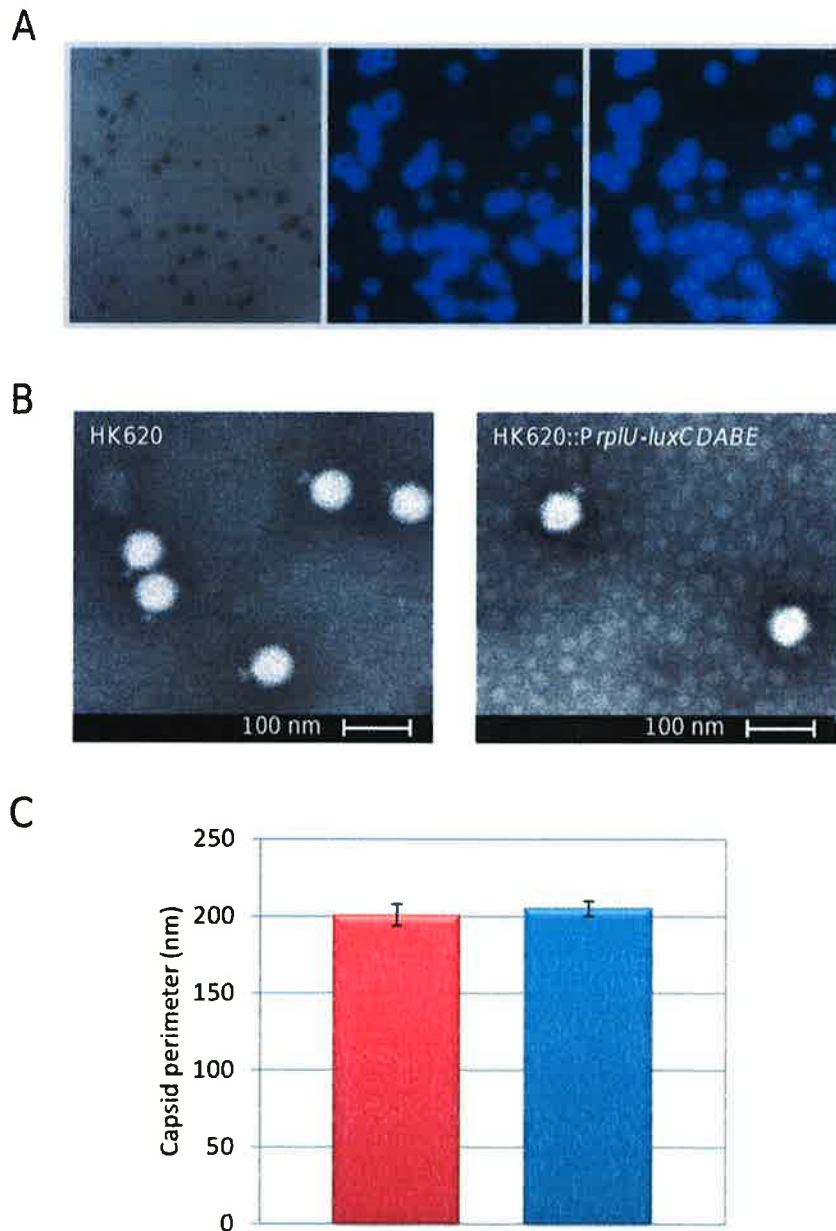


Figure 2. Luminescence emission upon infection with engineered HK620 phages. Bacterial growth (A) and luminescence profile (B) obtained from non infected *E. coli* TD2158 alone (blue), infected with WT HK620 (red) or recombinant phages HK620::*PrpoD-luxCDABE* (green), HK620::*PrpIU-luxCDABE* (purple) were compared.

2. Capsid structure of recombinant phages particles

The insertion of the entire *luxCDABE* operon together with the *PrpIU* promoter and a resistance cassette into the HK620 genome increased the genome size up to 120% compared to the WT HK620 genome. Considering the luminescence of the lysis plaques, each one arising from a single infecting recombinant phage, we obtained an homogenous population of recombinant phage (Fig. 3A). It thus looked that a vast majority of plaque forming units (pfu) integrated the whole construct. Several rounds of plaque isolation were performed to get an homogenous phage population. Therefore, we asked whether the packaging of large genome could modify the size and/or the shape of the capsid. We compared the perimeter and the sphericity of WT and recombinant HK620 capsids. Around hundred viral particles were analyzed using transmission electronic microscopy (see Material and Methods). Results show that recombinant phages

1 had capsid with icosahedral structures as the WT HK620 phage (Fig. 3B). The capsid perimeters were $205 \pm$
2 5 nm and 200 ± 8 nm for HK620::*PrpIU-luxCDABE* and WT HK620, respectively (Fig. 3C). These results
3 indicate that the recombination event did not affect the overall structure of the recombinant phages
4 although an important add-on to the genome has been made. At this stage we did not know if the capsid of
5 HK620 can tolerate such an increase in the genome size or if some dispensable parts of the genome are
6 spontaneously deleted and/or rearranged as suggested in other phage engineering work (Springman et al.,
7 2012). Only genome re-sequencing, which will be performed previous in field tests, will provide an answer.
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Figure 3. Characteristics of the HK620::*PrpIU-luxCDABE* prototype. (A) Lysis plaques under white light (left), chemiluminescence emission (middle), and overlay (right); (B) Electron micrographs of WT HK620 (left) and HK620::*PrpIU-luxCDABE* (right); (C) Capsid perimeter average of WT HK620 (red) and HK620::*PrpIU-luxCDABE* (blue). $n = 100$.

3. Engineering of HK97 and λ phage by insertion of *lux* operon

Phage HK620 strictly infects the *E. coli* strain TD2158 (Dhillon et al., 1998). We used this phage-host couple to develop our prototypes. A general objective of the method is to develop phage-based biosensors with either non overlapping specificities, or with large recognition spectra to detect various strains at the same time. We thus attempted to engineer *E. coli* K12 infecting phages. HK97 and λ phages specifically infect *E. coli* strains and use a *cos* system to package the DNA (Dhillon et al., 1980). The *cos* packaging system is described to be less flexible to variation in genome size than the *pac* packaging system (Campbell, 1994). In order to assess the impact of the insertion site on the stability of the engineered phage, we chose 2 intergenic regions devoid of transcription signals in HK97 and λ genomes as insertion sites (Fig. 1). One region consists of the intergenic region between two convergent genes *rz* and *bor* in λ genome, the other one corresponds to the region downstream gene *p56* in HK97. Briefly, we inserted easily the *PrpIU-luxCDABE* operon into the genome of these prophages (*i.e.* under a genome-integrated form) using the λ Red recombining technique (Fig. 1 and Material and Methods section). The insertion generated an increase in genome size of 20% for HK97 and 16% for λ , whose genome sizes are 39 kb and 48 kb, respectively. MMC treatment allowed the production and release of HK97::*PrpIU-luxCDABE* particles, and after 2 rounds of plaque purification, an homogeneously luminescent population could be obtained (Fig. 4). In contrast, although the genetic construction was easily obtained, no viable λ ::*PrpIU-luxCDABE* phage particle could be produced. These results indicate that although the *cos* system is known to be less tolerant than the *pac* one, it is not the only determinant that drives the success of large insertion engineering.

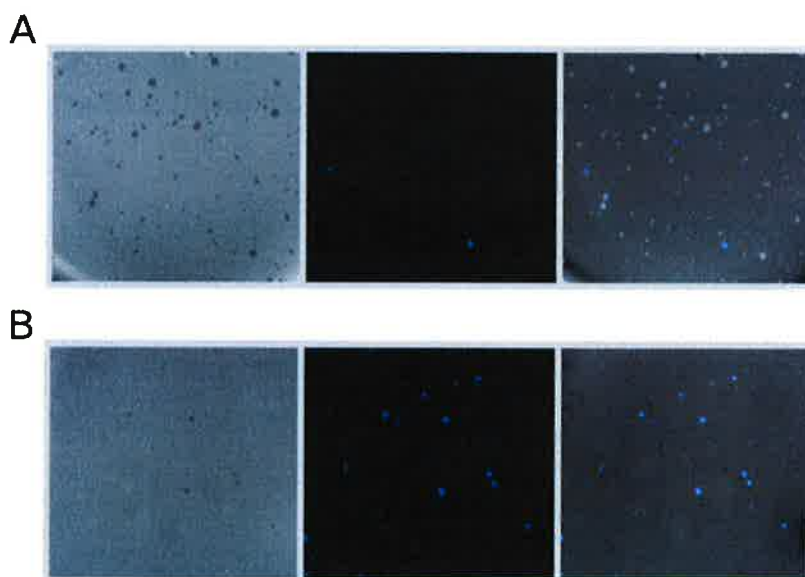


Figure 4. Enrichment of luminescent HK97::*PrpIU-luxCDABE* plaques (A) and (B) Lysis plaques under white light (left), chemiluminescence emission (middle), and overlay (right); (A) plaques obtained immediately post-MMC induction; (B) plaques obtained after two isolation rounds.

1 We then analyzed the luminescence emission profiles obtained from non infected *E. coli* MG1655, infected
2 with WT HK97 or recombinant phage HK97::PrpIU-luxCDABE (Fig. 5A). When *E. coli* MG1655 was infected
3 with the WT phage, the luminescence signal was homogenous and did not differ significantly from what
4 observed under non-infected conditions. In contrast, the luminescence rapidly increased during the first
5 hour of infection and reached a maximum around 10 hours post-infection with HK97::PrpIU-luxCDABE.
6 When comparing the luminescence obtained post-infection in strains MG1655 and TD2158, using two
7 different phages nonetheless carrying the same reporter fusion, we found that the signal was higher in
8 strain TD2158, indicating that the genetic background influences the emission of chemiluminescence (Fig.
9 5B, light curves), regardless of the CFU/ml present in the assay (data not shown). Moreover, we found that
10 the background level of luminescence was higher in MG1655 than in TD2158 (Fig. 5B, dark curves).
11 Therefore, considering these two strains, the ratio signal over noise is very different from one strain to
12 another, and this aspect will have to be taken into consideration for future developments.
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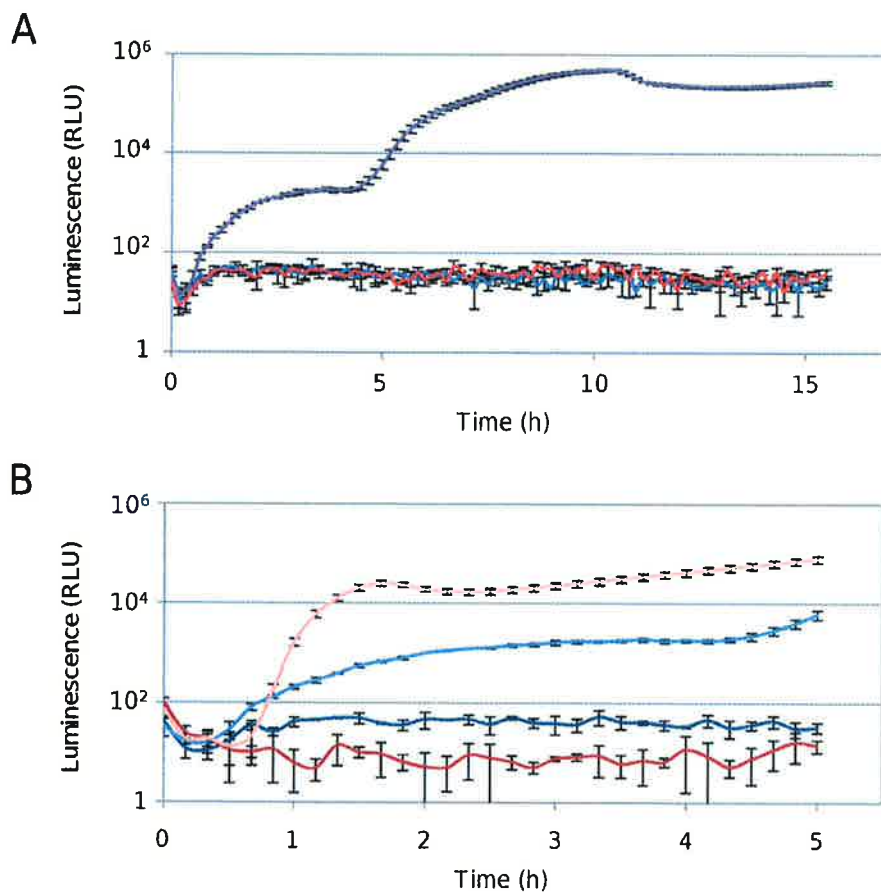


Figure 5. (A) Luminescence profile obtained from non infected *E. coli* MG1655 alone (blue), infected with WT HK97 (red) or recombinant phage HK97::PrpIU-luxCDABE (purple) were compared. (B) Luminescence profile obtained from MG1655 (blue) and TD2158 (red) alone (dark curves) or infected with recombinant phages HK620::PrpIU-luxCDABE or HK97::PrpIU-luxCDABE respectively (light curves) were compared for 5 hours.

4. Detection limit

1 In order to reach the limit of detection, samples at a concentration of 10^7 bacteria.ml⁻¹ were serially 10-fold
2 diluted in supplemented M9 medium before infection with HK620::*PrpIU-luxCDABE*. The luminescence
3 intensity was monitored every 10 min for 2 hours. The results illustrated in **Fig. 6A** indicated that the signal
4 generated 1.5 hours post-infection and after on, in a sample containing *E. coli* TD2158 cells at a
5 concentration of 10^4 bacteria.ml⁻¹ is higher than the basal luminescence. Therefore, we show that detection
6 of bacteria is possible using this tool. It is important to notice that the linearity of the detection is not
7 affected by the MOI since in this assay the MOI varies according to the density of the bacterial suspension.
8 We also tested the detection of *E. coli* TD2158 cells present in a tap water sample artificially contaminated
9 (**Fig. 6B**). An overnight bacterial culture was centrifuged and cells were resuspended in tap water. The
10 artificially contaminated sample was incubated at room temperature overnight to mimic an accidental
11 contamination. The spiked sample was serially 10-fold diluted in tap water then each bacterial sample was
12 infected with HK620::*PrpIU-luxCDABE*. The limit of detection obtained with HK620::*PrpIU-luxCDABE* in tap
13 water was around 10^4 bacteria.ml⁻¹. Thus, the sensitivity in tap water is comparable to what obtained in
14 supplemented M9 medium.
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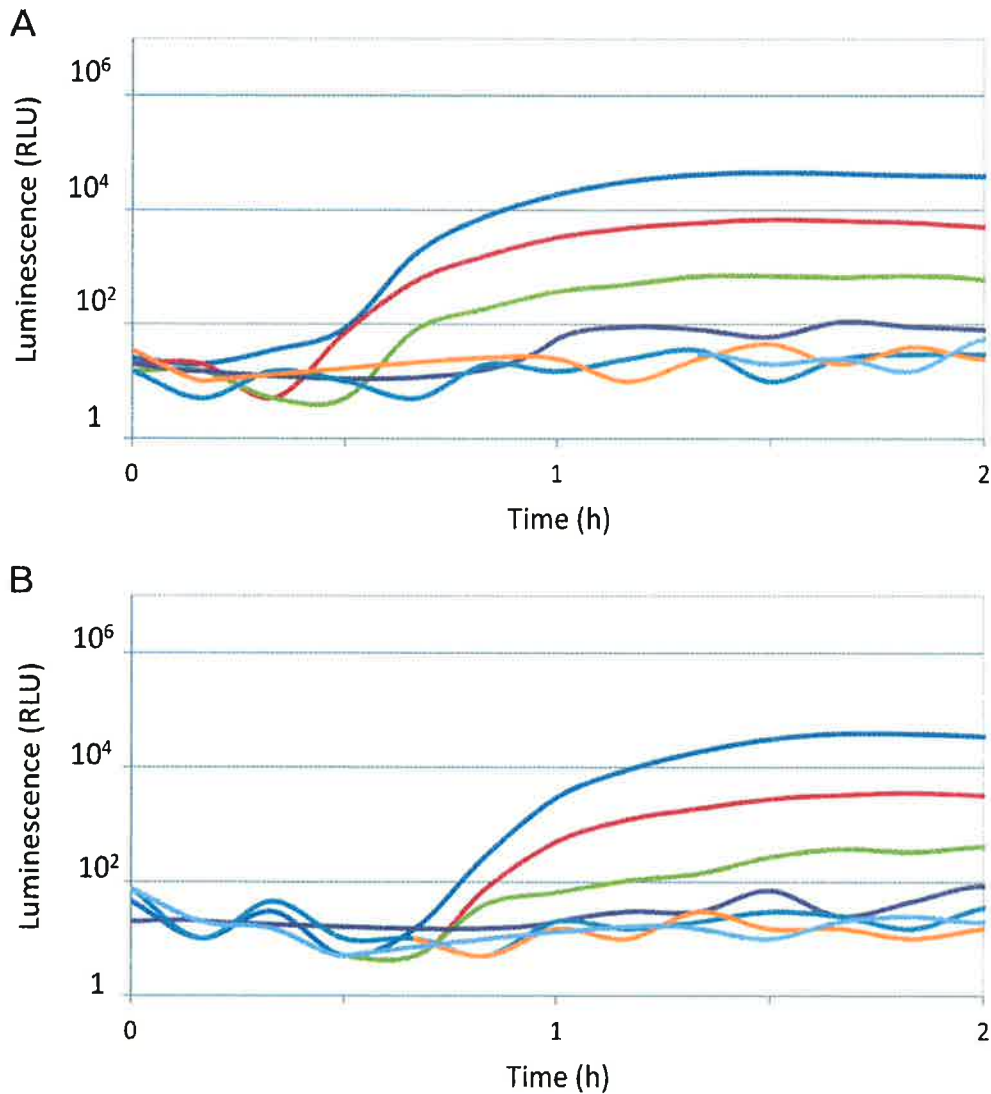


Fig. 6 Assay limits. (A) Determination of the minimal concentration of *E. coli* TD2158 that can be detected. Serial 10-fold dilutions ranging from 10^7 to 10^1 bacteria. ml^{-1} were infected with HK620::*PrpIU-luxCDABE* phage. Infected cells were incubated at 30°C . Luminescence was monitored each 10 min using a microplate reader. (B) Detection in a spiked tap water sample. *E. coli* TD2158 in stationary phase was added to a tap water sample. After an overnight incubation at room temperature, the sample was infected with HK620::*PrpIU-luxCDABE*. Luminescence intensity was monitored every 10 min. Color code: 10^7 bacteria. ml^{-1} = dark blue (MOI= 10^1), 10^6 = red (MOI= 10^2), 10^5 = green (MOI= 10^3), 10^4 = purple (MOI= 10^4), 10^3 = turquoise (MOI= 10^5), 10^2 = orange (MOI= 10^6), 10^1 = light blue (MOI= 10^7).

III. Materials and Methods

1. Bacterial strains, bacteriophages and media used in this study

Bacterial strains and bacteriophages used in this study are listed in Table 1. The presence of the HK620, λ and HK97 prophages in strains TD2158 and MG1655 was checked by PCR amplification of the *att* sites using dedicated primer pairs (Table 2). When necessary ampicillin ($100 \mu\text{g}.\text{ml}^{-1}$) or kanamycin ($40 \mu\text{g}.\text{ml}^{-1}$) were

1 added to growth media. LB medium was used for lysogen growth and infections. When necessary, 1.5%
2 (hard) or 0.75% (soft) of agar was added to the growth medium. M9 minimal medium supplemented with
3 casamino acids (0.2%), D-glucose (0.3%), vitamin B1 (1 mg.ml⁻¹), MgSO₄ (0.2 mM), CaCl₂ (0.1 mM) was used
4 for luminescence detection assays.
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6 **2. Phage engineering**

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9 Recombinant prophages were constructed by the insertion of *luxCDABE* (Gene bank accession number:
10 M90093.1) and kanamycin resistance genes into intergenic regions of HK620, λ and HK97 prophages using
11 the lambda Red homologous recombination method (Datsenko and Wanner, 2000). PCR fragments used as
12 the lambda Red homologous recombination method (Datsenko and Wanner, 2000). PCR fragments used as
13 substrates for homologous recombination are described in Fig 1. They contain the *luxCDABE* operon under
14 the control of either *PrpIU* or *PrpoD* promoters and the kanamycin resistance-cassette, flanked by 80 bp of
15 homology with the integration region. To construct the *PrpIU-luxCDABE* plasmid, the *PrpoD* promoter of
16 plasmid *PrpoD-luxCDABE* (Preveral, S. et al., in this issue) was exchanged with the *PrpIU* promoter using
17 EcoRI and NcoI sites. PCRs were performed using the high fidelity DNA polymerase PrimeSTAR Max Premix
18 2X (Takara), plasmids *PrpoD-luxCDABE*, *PrpIU-luxCDABE* (1 ng. μ l⁻¹) as templates and primer pairs listed in
19 Table 2. PCR fragments purified using the QIAquick PCR purification Kit (Qiagen) were treated with the
20 restriction enzyme DpnI (Takara) in order to eliminate the template plasmids. After a purification step using
21 the MinElute reaction cleanup kit (Qiagen), 1.5 μ g of PCR fragments were electroporated into
22 LCB6205(HK620)/pKD46, MG1655(λ)/pKD46 and MG1655(HK97)/pKD46 using the Eporator electroporation
23 system (Eppendorf). Luminescent colonies were selected with a luminescence imager (LAS4000, GE
24 Healthcare). The integration of the constructs was checked by PCR using flanking primers. Two isolation
25 rounds were performed to select for recombinant lysogenic bacteria only.
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38 **3. MMC induction, production and purification of recombinant phages**

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41 Recombinant phages were collected following MMC induction (5 μ g.ml⁻¹) under shaking for 3 hours. Then,
42 cells were treated twice with chloroform and pelleted by centrifugation at 4,500 g for 10 min, the
43 supernatants were filtered (0.22 μ m) and stocked at 4°C. For phage production, 100 μ l of phage suspension
44 (around 10⁹ PFU.ml⁻¹) and 100 μ l of an overnight host culture were mixed with 3 ml of LB soft agar and
45 spread onto a LB plate. After incubation overnight at 37°C, the top agar layer was collected and mixed with
46 10 ml of LB. The mix was left standing for 30 min to allow phage diffusion into the medium and treated
47 twice with chloroform as described above. Phages were then purified by centrifugation at 20,000 g for 1
48 hour. Phage pellets were resuspended in Tris-HCl 50 mM pH 8.0, NaCl 100 mM, buffer and incubated
49 overnight at 4°C under shaking. Purified phage suspensions were titered and kept at 4°C. To reach
50 homogeneity in the engineered phage population several round of plaque isolation were sometimes
51 necessary. Briefly, instead of recovering the entire soft agar layer, individual plaques were recovered and
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isolated on a new soft agar plate. Lysis plaques were then observed to check the luminescence of individual plaques.

4. Optical density and luminescence measurements post-infection

To monitor growth of the bacteria upon infection, the OD₆₀₀ was measured as a function of time. An overnight culture was diluted at a initial OD₆₀₀ of 0.1 in 1 ml of LB medium and the appropriate volume of phage suspension was added to reach a multiplicity of infection (MOI) of 10. 170 µl of the mixture were added to a microplate with clear bottom (CELLSTAR®, 96 well Microplate, White/µClear®, Greiner bio one) and incubated at 30°C without agitation. OD₆₀₀ was measured every 10 minutes for 12 hours. Luminescence monitoring was performed the same way except that white microplates were used (CELLSTAR®, 96 well Microplate, all White, Greiner bio one). Luminescence was monitored using the Infinite® M200 (Tecan) system. For the determination of the limits of detection, experiments were carried out in supplemented M9 medium and we used a single concentration of phage added to the diluted bacterial suspension, therefore the MOI varied according to the density of the bacterial suspension.

5. Electronic microscopy

Electronic microscopy was performed at the Mediterranean Institute of Microbiology (IMM) facility. Phage suspensions (5 µl) were dropped onto a copper grid covered with Formvar and carbon and left 3 min. at 25 °C, after what the excess of liquid was removed. Phage particles were stained according to (Ackermann, 2009) with a 2% uranyl acetate solution. Dried grids were then observed using a transmission microscope FEI Tecnai 200 kV coupled to a Eagle CCS 2kx2k camera. Images were then analyzed using the ImageJ software.

IV. Conclusions

The major requirement of the COMBITOX project was to adapt our biodetection system based on bacteriophage engineering to luminescence detection (Ansaldi et al., 2015). In order to design a substrate independent biodetector, we used as a reporter system the entire *luxCDABE* operon that encodes a luciferase as well as the substrate generating system. The engineering step, although very challenging given the small size of bacteriophage genomes, was successful since we managed to engineer two functional phages HK620 and HK97 that have different packaging systems and without removing any part of the respective phage genomes. Our results indicate that *lux* phages could be used to detect around 10⁴ bacteria/ml, which is only two log apart from the limit imposed by the EU regulation (European directive 2006/7/EC of the European Parliament and of the Council of 15 february 2006 concerning the management of bathing water quality and repealing Directive 76/160/EEC). Further improvements, in particular a luminescence detection device with a higher sensitivity, such as developed in (Descamps, E. et al., in this

issue) are needed to reach the European guidelines. The design and development of substrate independent phage-based biosensors will be pursued in order to detect Enterobacteriaceae strains commonly found as pollutants in water distribution networks. A key issue will be to detect enteric bacteria after a prolonged stay in water. Indeed, upon an accidental human or animal contamination, most bacteria present in the environment are in a stationary phase or are considered as viable but not cultivable (VBNC) (Li et al., 2014). In a previous work, we showed that *gfp* versions of our prototypes were suitable to detect *E. coli* cells incubated 24 h in sea water (Vinay et al., 2015). Once the luminescence detection will be improved to match the regulation, appropriate assays will be performed with the luminescent versions of the phage-based biosensors to detect bacteria under prolonged stationary phase condition or VBNC bacteria.

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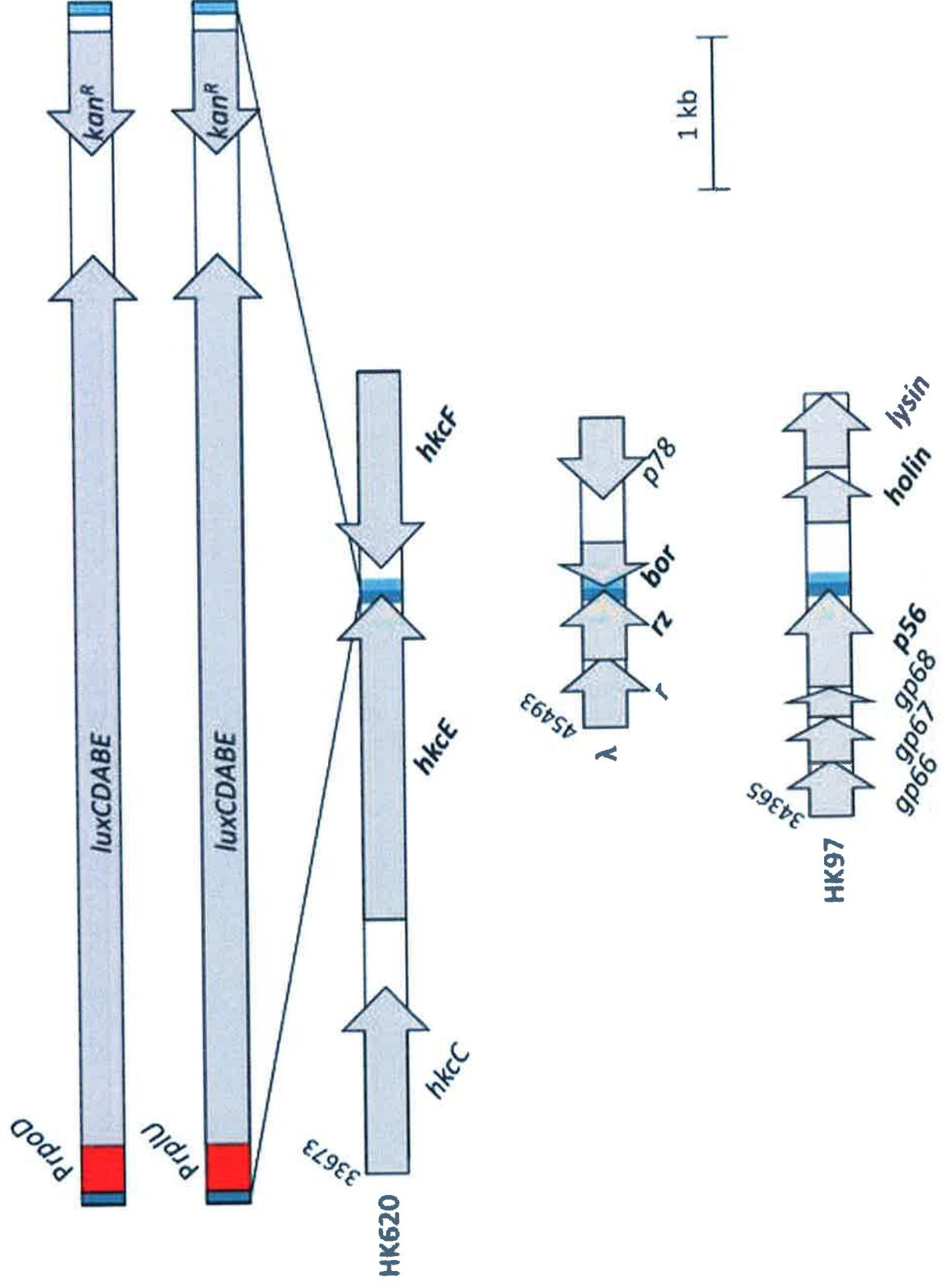
Table 1. *E. coli* strains and bacteriophages used in this study

Strains	Characteristics	References
TD2158	Environmental <i>Escherichia coli</i> H strain	(Dhillon et al., 1998)
LCB6205	Phage-free <i>Escherichia coli</i> TD2158, previously named <i>E. coli</i> TD2158_PL4	(Menouni et al., 2013)
LCB6205(HK620)	LCB6205 lysogen for HK620	(Vinay et al., 2015)
LCB6146	LCB6205(HK620 <i>hkcEF::PrpoD-luxCDABE</i>), Km ^R	This work
LCB6147	LCB6205(HK620 <i>hkcEF::PrpIU-luxCDABE</i>), Km ^R	This work
MG1655	Reference <i>Escherichia coli</i> K12 strain	(Blattner et al., 1997)
MG1655(HK97)	MG1655 lysogen for HK97	M.A. Petit
LCB6188	MG1655(HK97 <i>p56-holin ::PrpIU-luxCDABE</i>), Km ^R	This work
MG1655(λ)	MG1655 lysogen for λ	Lab' collection
Bacteriophages		
HK620	Temperate bacteriophage specifically infects <i>E. coli</i> TD2158	(Clark et al., 2001; Dhillon et al., 1998)
HK620 <i>hkcEF::PrpoD-luxCDABE</i>	Recombinant phage induced from LCB6146	This work
HK620 <i>hkcEF::PrpIU-luxCDABE</i>	Recombinant phage induced from LCB6147	This work
λ	Temperate bacteriophage, infects <i>E. coli</i> K12	(Hendrix, R et al., 1983)
HK97	Temperate bacteriophage, infects <i>E. coli</i> K12	(Juhala et al., 2000)
HK97 <i>p56-holin ::PrpIU-luxCDABE</i>	Recombinant phage induced from LCB6188	This work

Table 2. Plasmids and primers used in this study

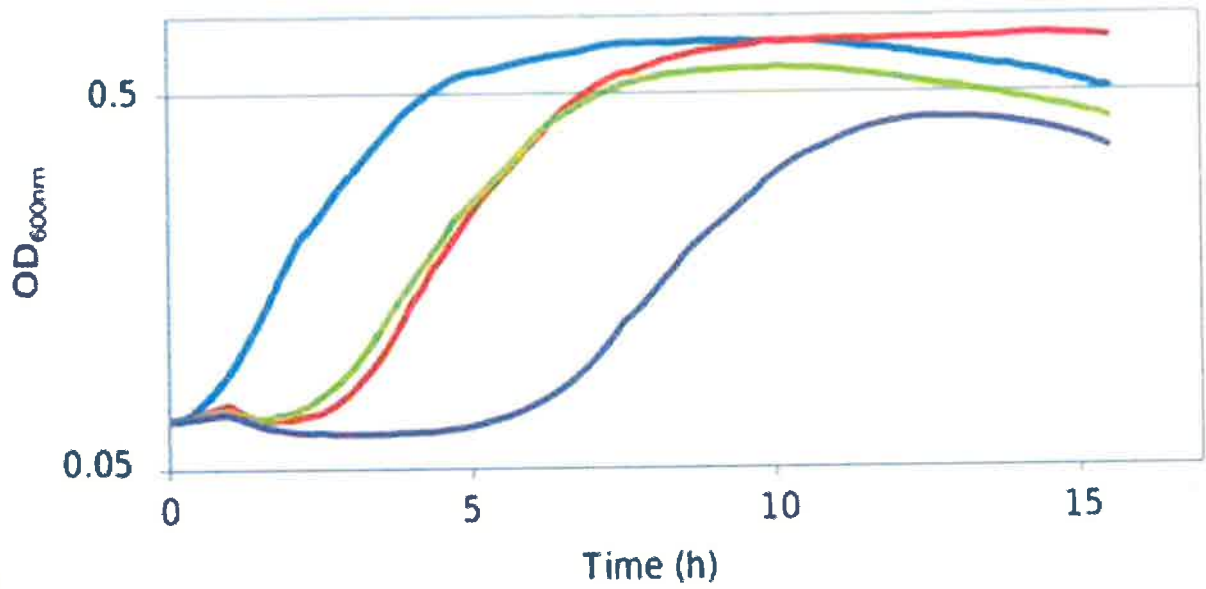
Plasmids	Characteristics	References
pKD46	lambda Red expression plasmid. <i>bla</i> , <i>araC</i> , <i>gam</i> , <i>bet</i> , <i>exo</i> genes; <i>P_{bad}</i> promoter ; ori R101	(Datsenko and Wanner, 2000)
<i>PrpIU-gfp</i>	pUA139 containing the <i>PrpIU</i> promoter region	E. Bouveret
<i>PrpoD-luxCDABE</i>	p15A origin, kan ^R	(Prévéral et al., 2016)
<i>PrpIU-luxCDABE</i>		This work
Primers	Sequences ^a	References
pRplU-EcoRI-Rev	CGGAATTCGTGTCCAGCTTTCCAGGC	
NcoI-pUA66-Fow	CAATCCATGGGAGGCCCTTCGTCTTCAC	
attL-ter	CATCGAGAAGGCGGTATGGTTTTTC	(Panis et al., 2010)
attL-pro	AATGGATATAACGAGCCCCTCC	(Panis et al., 2010)
attL-Lambda-Fwd	ACCAGGCGCGGTTTGATCAG	
attL-Lambda-Int-Rev	TTCACGAGTTGCGCAGTTTG	
attR-Lambda-Rev	GCGCAATGCCATCTGGTATC	
pRpoDlux_hkcEF_Fow	G TTCAGTTAGTGTGAGGTTGATGCTGCAGCGTCA G GGTGTGAGTATTTGATATCTACGAAATAATGTGC T TTTGCATAATTTCCATGGGCATACTTGATGA	
pRpoDlux_hkcEF_Rev	A AATATCAATTACATCTTTCTGTTTTATGGTGTGAC C ATTACATGCTTTTATATAAACAATTCTTCTTGGT T TATCATGTATCAACAAAGCCACGTTGTGT	
pUA66_hkcEF_Fow	G TTCAGTTAGTGTGAGGTTGATGCTGCAGCGTCA G GGTGTGAGTATTTGATATCTACGAAATAATGTGC T TTTGCATAATTTGAGGCCCTTCGTCTTCAC	
hkcE_dir	TTGGTTGGGGTTGGGTATAA	(Vinay et al., 2015)
hkcE_down_rev	CACCACGAGGTGGTTAAAGC	(Vinay et al., 2015)
pUA-Lambda-Rz-Bor-F	A GAGGCTGATCACTATGCAAAAACAACCTGGAAG G AACCAGAAGTATATTAATGAGCAGTGACAGAT A GAGTTGCCATATGGAGGCCCTTCGTCTTCAC	
pLUX-Lambda-RZ-Bor-R	C GGTTTTATTACTTTAGGCATTTATACTCCGCTGG A AAGCGCGTGTGTATTGCTCACAATAATTGCATGA G TTGCCATCGTCAACAAAGCCACGTTGTGT	
pUA-HK097-p56-Holin-F	G AAGAGTCAATTGCAGACAACATTTGAACGCG G TCCACACGTTAGCAGCATGATTGCCACGGATGG C AACATATTAACGGGGAGGCCCTTCGTCTTCAC	
pLUX-HK097-p56-Holin-R	T CAGGGCTTTATTTAACGAGTGCATTTATCCATCG T TGATGTCAAATTTACCCAATTTATTCAAAAAGT C CAATATCATGTCAACAAAGCACGTTGTGTC	
Bor-lambda-Rev	GGCAGCAGTAGCACCAAAGG	

^a sequences in bold correspond to priming sequences that allow the amplification of the PCR fragments used for recombination.

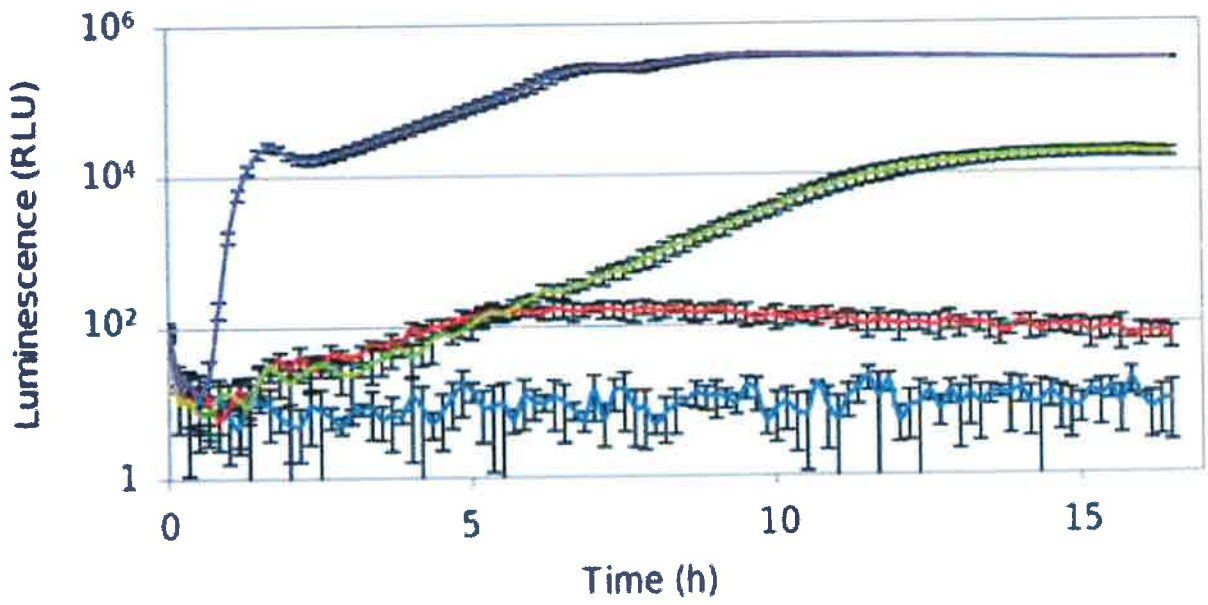


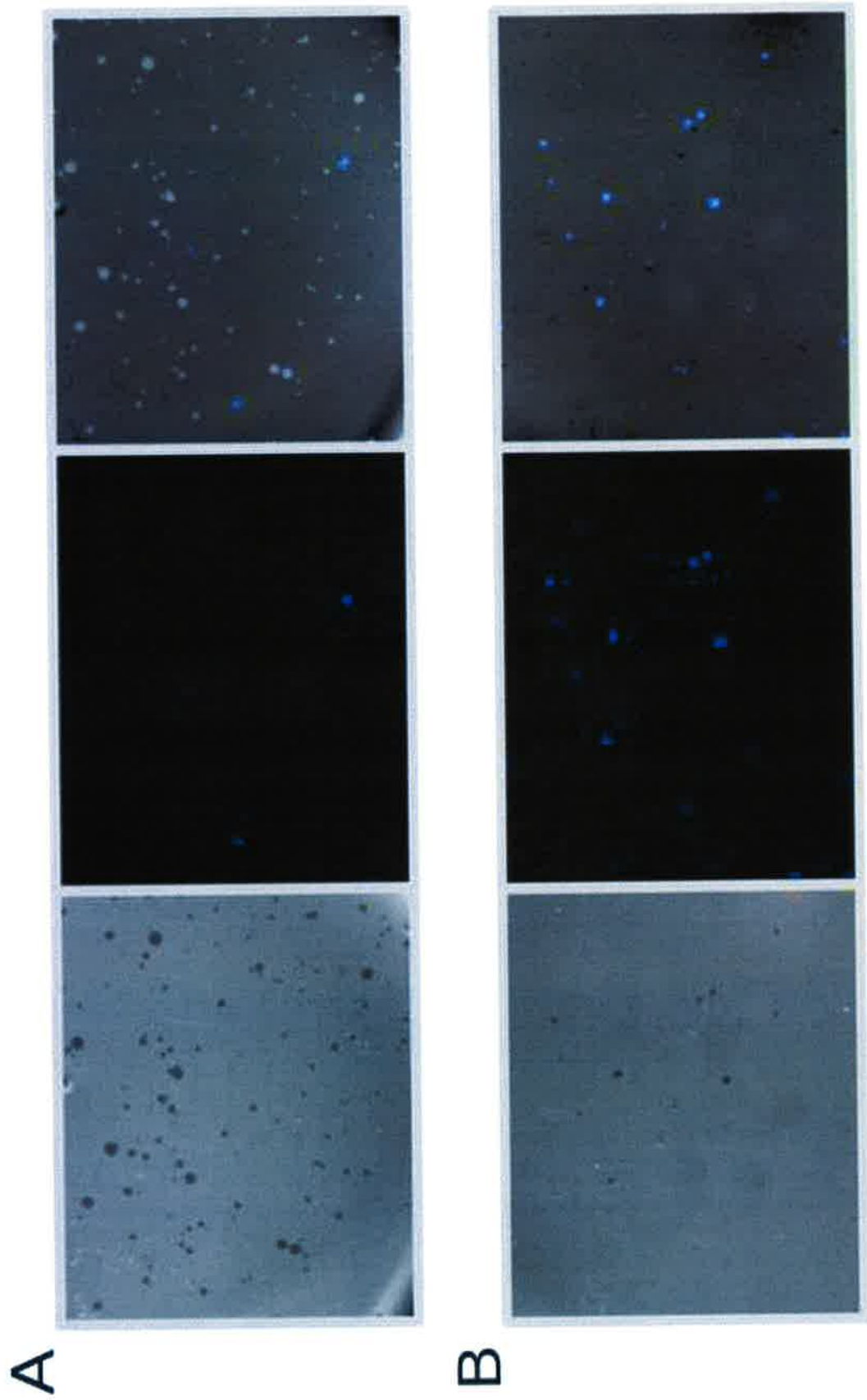
Figure

A



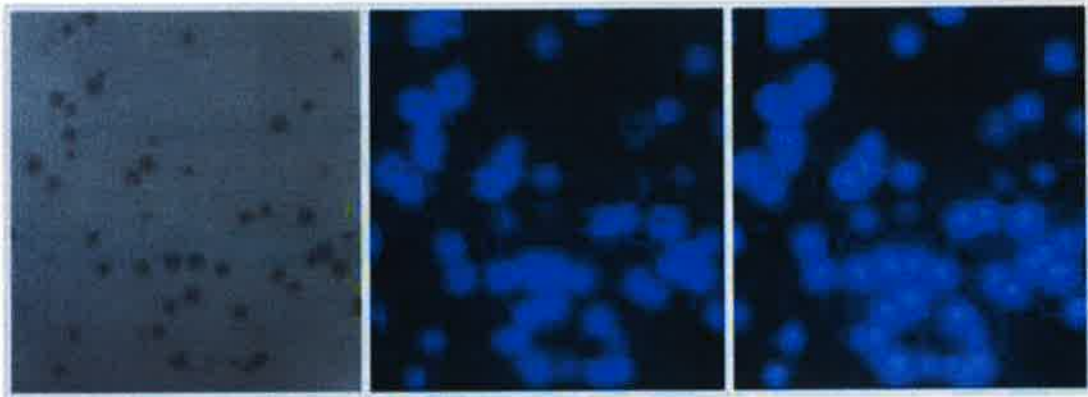
B



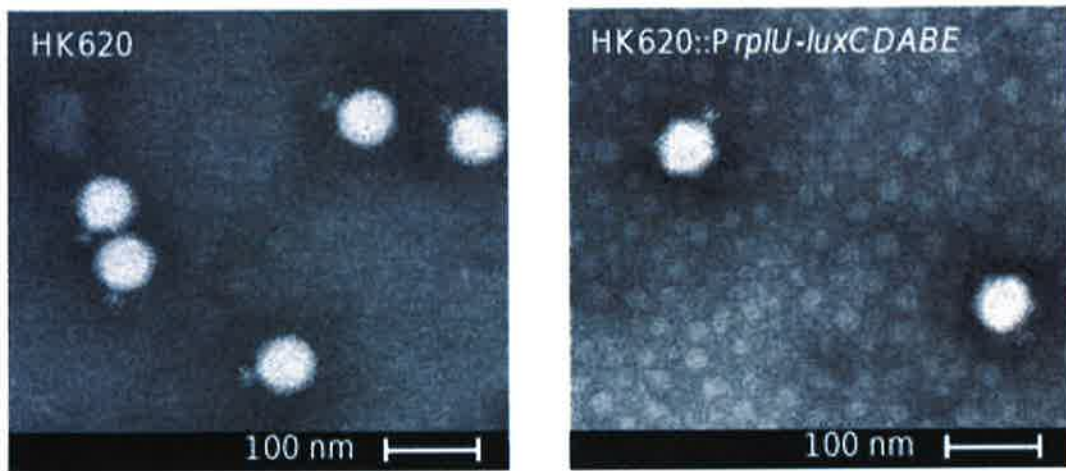


Figure

A



B



C

