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# Induction and elimination of prophages using CRISPR-interference

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**Abstract**

Prophages are widely spread among bacterial genomes, and they can have positive or negative effects on their hosts. A key aspect in the study of prophages is the discovery of their induction signals. Prophage induction can occur by inactivating a phage transcriptional repressor, which is responsible for maintaining the lysogenic state. This repressor can be inactivated through the bacterial SOS response. However, the induction signals for numerous prophages do not involve the SOS system, and therefore significant efforts are needed to identify these conditions. Similarly, curing bacterial strains of inducible prophages is a tedious process requiring the screening of several colonies. Here, we investigated whether transcriptional silencing of a prophage repressor using CRISPRi would lead to prophage induction (CRISPRpi). Using *E. coli* phages λ and P2 as models, we demonstrated the efficiency of CRISPRi for prophage induction and for curing lysogenic strains of their prophages.

## Introduction

Bacteriophages (or phages) are viruses that infect bacteria. These bacterial viruses can be either virulent or temperate. Virulent phages are only able to infect a bacterial cell through a lytic cycle, which leads to the lysis of the infected cell and the release of newly produced virions. Temperate phages can replicate in a similar manner or perform a lysogenic cycle, during which the phage enters in a latent state. During lysogeny, the viral genome is either integrated into the bacterial chromosome or remains as an episome in a plasmid-like state and are designated as prophages.[1–3]

Prophages are present in at least 50% of bacterial genomes.[4,5] They are major drivers of the genetic diversity among species and greatly contribute to the bacterial pangenome.[6] Lysogeny can be costly for the host bacteria compared to non-lysogenic bacteria.[7,8] However, prophages can also carry genes that increase bacterial fitness, in a phenomenon known as "lysogenic conversion". These extra genes are often referred to as moron genes. They can provide protection against other phages,[9] increase bacterial fitness,[10] make the bacteria more resistant to external stresses[11,12] and even carry virulence genes to enhance the bacteria's ability to survive in an infected host.[13-16] The identification of these moron genes is difficult because they are only expressed during the lysogenic state and most of them have unknown function. The simplest way to identify the impact of these genes is to delete the entire prophage from the bacterial chromosome and search for phenotypes of the prophage-free derivative.[10,11,15] The bacterial strain can be cured of a prophage through induction using mitomycin C or UV light [11,17,18] but this is a very-time consuming process as several conditions and colonies need to be tested. Prophages can also be removed through recombination using genetic tools, such as a suicide plasmid or using CRISPR-Cas9, but with the risk that the deletion does not restore the ancestral DNA sequence.[10,19–21] Furthermore, it is difficult to assess the efficiency of theses protocols for prophage curing as limited data is available.

For the majority of temperate phages, lysogeny is maintained through a simple yet incredibly efficient mechanism. They exhibit a typical genetic organisation in their lysogenic module (Fig. 1A), where two transcriptional regulator genes, the repressor and the activator are on opposite sides of transcription. The promoters of the repressor and activator are usually located in the intergenic region between these two genes.[22,23] Whether a prophage can maintain its lysogenic state is dictated by the presence or absence of a repressor protein within the cell. By binding to specific operator sequences that overlap the promoters, the repressor prevents the transcription of genes that are involved in triggering the lytic cycle and controls its own expression (Fig. 1A). [24] The repressor gene is the only gene that can be transcribed (sometimes transiently) during lysogeny, with the exception of moron genes.[25] The number of repressors in the cell is tightly regulated and it determines the stability of the prophage in the bacterial genome during bacterial division.

Prophages switch to the lytic cycle when there is an induction signal in the lysogenic cell or when there is a spontaneous induction. Prophage induction is allowed when the repressor is supressed from lysogenic cell. This can be achieved by activating the host's SOS system,[26] a stochastic event,[27] or a reduction in the ability of the repressor to bind its binding sequences.[28,29] While a number of induction signals have been described,[29] the most frequently observed is the activation of the host SOS pathway, triggered by DNA damage.[30] An induction signal usually leads to the proteolysis of the phage repressor, allowing the transcription of genes that are involved in the lytic cycle. Prophages can also be induced spontaneously because of the transcriptional background noise, which can block the production of the repressor, as observed with prophage λ in a *ΔrecA E. coli* strain.[24] This process leads to the presence of free virions in the supernatant of non-induced lysogenic cultures, or in bacterial mutants with an inactive SOS system.[24,30] When searching for novel prophages, whether the prophage is able to spontaneously produce virions is usually determined first. The second step is to determine whether the

prophages can be induced. Discovering a prophage induction signal requires a significant amount of effort, and these efforts do not always yield positive results.[31–33] Early techniques to artificially induce prophages selected for thermoinducible mutants, but the generation of these mutants is also time consuming.[34] An ideal way to induce prophages would be to impede the transcription of the prophage repressor.

CRISPR-interference (CRISPRi) is a derivative of the CRISPR-Cas9 genome editing technology. It is based on a SpyCas9 mutant called Dead Cas9 (dCas9).[35] Two mutations in Cas9 inhibit its endonuclease activity without affecting its ability to form a DNA recognition complex by binding with the guide RNA (sgRNA) and the *trans*-acting RNA (tracrRNA). The complex binds to the targeted nucleotide sequence, but dCas9 complex does not cut the targeted DNA and remains bound to the targeted sequence. This system can be used as an artificial transcriptional repressor by targeting promoters or sequences located inside the gene itself.[36]

Here, we describe how functional prophages can be induced by inhibiting transcription of the repressor gene using CRISPRi. We show that the λ and P2 prophages of *E. coli* can be induced using CRISPRi. In addition, the significant bacterial mortality resulting from prophage induction facilitates the recovery of surviving prophage-cured bacteria.

## Materials and Methods

### Bacterial strains, growth media and plasmids

The bacterial strains and phages used in this study are listed in Supplementary Table S1. *E. coli* strains were grown at 37°C in TSB (Tryptic Soy Broth, Difco) medium or Lysogenic Broth (LB) (10 g/L Bactotryptone, 5 g/L Yeast Extract, 10 g/L NaCl). Solid media were supplemented with 1% agar (Difco) for the bottom plate and 0.75% agar for the top agar. The following antibiotic concentrations were used: 25 µg/mL chloramphenicol and 60 µg/mL

spectinomycin. Plasmids used in this study are listed in Table S2. Plasmid pCRISPathBrick was gifted by Mattheos Koffas (Addgene plasmid #65006; http://n2t.net/addgene:65006; RRID: Addgene_65006) and pFD116 was gifted by David Bikard (Addgene plasmid #124769; http://n2t.net/addgene:124769; RRID: Addgene_124769).

Spacer design and cloning into pCRISPathBrick and pFD116

The spacers were chosen based on several criteria. The matching protospacers should overlapped the promoter sequence of the targeted gene or the starting 5' sequence of the gene. They have to be immediately adjacent in their 3'-end to a PAM (5'-NGG-3'). The spacers are 30 nt-long for pCRISPathBrick and 20 nt-long for pFD116. If possible, protospacers should be located on the same DNA strand as the promoter sequence. The last 10 bases of the spacer and its PAM were blasted (BlastN) against the genome of *E. coli* strains NEB5-alpha, K12 and C-2322 to ensure specificity. Primers were ordered with appropriates extension for cloning into pCRISPathBrick and pFD116. Spacer cloning into the different plasmids was constructed as previously described.[37-39] The list of primers used is presented in Table S3. The ligation reactions were electroporated in *E. coli* NEB5-alpha. The cloning of the appropriate spacers into the plasmids were confirmed by PCR and sequencing.

PCR protocol and gel migration

PCR was performed using Bio Basic Taq polymerase and thermocycler Eppendorf Mastercycler® Nexus X2, according to the manufacturers' recommendations. PCR products were visualized using BET coloration on 0.8% or 2% agarose. Primers used for PCR amplification are also listed in Table S3.

Electro-competent cells and transformation

Transformation of *E. coli* was performed according to the following protocol. *E. coli* cultures were grown overnight at 37°C in TSB medium and then diluted 1/100-fold dilution in 150 mL of TSB medium, followed by incubation at 37°C with agitation (200 rpm). When the $OD_{600nm}$ reached between 0.5 and 1.0, cells were placed on ice for at least 30 minutes. Cells where then centrifuged at 10,000 x g for 12 min at 4°C, resuspended in 75 mL of deionized cold-sterile water, and centrifuged again. Cells were resuspended in 2 mL of cold-sterile 10% glycerol, and centrifuged in a tabletop centrifuge at 12,000 x g for 2 min. Finally, cells were resuspended in 300 µL cold-sterile 10% glycerol. For electroporation, 50 µL of competent cells were mixed with 100 ng of the appropriate plasmid in a cold electroporation cuvette (0.2 cm gap, BioRad), electroporated (2.5 kV, 200 Ω, 25 µF) and immediately resuspended in 950 µL of warm TSB (37°C). The electroporated culture was incubated at 37°C (200 rpm) for an hour of recovery and plated at the appropriate dilutions with antibiotics to obtain isolated colonies.

Prophage induction and curing experiments

Lysogenic strains for λ or P2 were prepared for electroporation as described above. After electroporation, cells were incubated at 37°C with agitation (200 rpm) for 1 hour. At the end of the incubation period, 50 µL of the transformed culture were added to 10 mL of TSB, supplemented with chloramphenicol. At the same time, appropriated dilutions were plated for isolation and quantification of prophage-cured bacteria by PCR. Cultures were grown overnight at 37°C with agitation (200 rpm). Then, cells were centrifuged, and the supernatant filtered (0.45 µm). Phage production was estimated by titrating the supernatant.

For prophage induction experiments, mitomycin C (1 µg/ml) was added 1 hour after cell recovery from the electroporation. Bacteria were then plated at the appropriate dilutions to recover isolated colonies. At the same time, 50 µL of the induced bacteria were diluted in 10

mL of TSB and incubated overnight at 37°C wit agitation (200 rpm). The day after, the cultures were centrifuged, the supernatant filtered (0.45 μm), and the phage titers determined.

Phage titration

Phage titers in bacterial supernatant were determined by using plaque assays. Indicator strains were grown overnight at 37°C (200 rpm) in LB medium supplemented with 0.2% maltose in the case of *E. coli* HER 1037. One-hundred microliters of the culture was then mixed with 100 μL of the appropriate dilution of phage lysate. The mix was then added to 3 mL of melted LB Top Agar (supplemented with 10 mM $MgSO_4$) and poured onto an LB plate. Once dry, the plates were incubated overnight at 37°C.

## Results

### Targeting the repressor of prophage λ with CRISPRi triggers its induction and curing

To determine the efficacy of prophage induction by targeting the prophage repressor using CRISPRi (Figure 1B), a set of experiments were performed with the *E. coli* strain K12 C600 that contains the prophage λ (HER 1025). This phage-host pair was selected because the lysogeni cycle of λ has been studied for decades and information is widely available for the regulation of *cI* gene expression via its promoter and the operator sequences.[23] The cI protein impedes the transcription of *cro* (the phage protein starting the lytic cycle) in two different ways: i) the cI protein binds to the operator regions *oR2/oR3*, overlapping the -10/-35 boxes of the pR promoter that is responsible for *cro* transcription (Figure 1C); ii) when cI proteins bind to *oL1/oL2* and *oR1/oR2*, they form a DNA loop that represses the pR promoter while enabling a small transcription of pRM, allowing synthesis of cI proteins. Since gene silencing can be achieved by inhibiting transcription initiation (by targeting the promoter) or by blocking

transcription elongation (by targeting the gene itself), we designed two CRISPR spacers to inhibit *cI* transcription: cI-1 and cI-2 (Figure 1C). Specifically, the spacer cI-1 targets the -35 promoter box of the *cI* gene, which overlaps the operator regions (*oR1* and o*R2*), while the spacer cI-2 directly targets the beginning of the *cI* gene (Figure 1C). A third spacer ea47 was designed as a control to target a prophage gene not involved in prophage induction (Figure 1C). This third spacer targets the promoter sequence of *ea47*, a non-essential gene for the λ lytic cycle that is not expressed in the prophage state.[40,41]

Two different vectors that carry CRISPRi were used. The first was the vector pFD116 [37] in which dCas9 expression was under the control of the TetR promoter. Despite the numerous colonies that were obtained after the transformation of *E. coli* strain HER1025 with the pFD116 vector carrying the cI-targeting spacers, none of them was able to grow in liquid medium, even in the absence of an inducer. There was a clear observation of cell lysis in the liquid culture. We hypothesised that this was due to the leaky expression of the TetR promoter [37], which was likely sufficient to trigger prophage induction and cell lysis.

The second plasmid used was pCRISPathBrick,[38] in which dCas9 is constitutively expressed. The pCRISPathBrick carries a default spacer that was designed to not target any sequences in the *E. coli* chromosome. Once all spacers were cloned individually in pCRISPathBrick, plasmids were transformed individually into *E. coli* HER1025 by electroporation. Because CRISPRi is constitutively expressed, we expected to observe a lower transformation efficiency when the bacteria were transformed with CRISPRi targeting *cI*. The transformation efficiency with the non-targeting spacer (nt-CRISPRi) reached an efficiency of $4.7 \times 10^7$ transforming cells/μg of DNA (Figure 1D), and a similar efficiency ($3.7 \times 10^7$) was measured when targeting *ea47*. A 1,000-fold decrease in the transformation efficiencies. ($3.1 \times 10^4$ and $1.2 \times 10^5$, respectively) was observed with the two CRISPRi constructs targeting either

199 the *cI* promoter or *cI* itself. These observations confirm that targeting a prophage repressor leads
200 to substantial bacterial mortality, possibly due to prophage induction.

201 In order to demonstrate λ induction, 50 µl of each transformation reaction after 1h of
202 recovery was added to 10 mL of TSB supplemented with chloramphenicol and incubated
203 overnight. Phage titer in the culture supernatant was measured the following morning (Figure
204 1E). When *E. coli* was transformed with the non-targeting or the *ea47*-targeting CRISPRi
205 plasmids, the phage λ titers were 2.0 x $10^6$ and 2.9 x $10^5$ PFU/mL, respectively. However, the
206 phage titers increased at least 100-fold when *E. coli* was transformed with a CRISPRi construct
207 targeting the *cI* gene, 7.5 x$10^8$ for cI-1 and 1.6 x $10^8$ PFU/mL for cI-2 vs 2.0 x $10^6$ PFU/mL for
208 controls. It should be noted that a low titer of phages was detected when no plasmids were
209 transformed in the cells (7 x $10^2$ PFU/mL). The low amount of phages is due to the absence of
210 an antibiotic resistance gene in the strain, which prevented bacterial growth in presence of the
211 antibiotic, thereby limiting the number of host cells. To confirm that the λ titers were solely due
212 to prophage induction and not the amplification of a λ ultravirulent mutant,[42] the culture
213 supernatant was spotted on the lysogenic strain HER 1025 and no lysis plaques were observed.
214 Taken altogether, we conclude that targeting the *cI* promoter or the *cI* gene using CRISPRi
215 triggers λ induction.

216 Interestingly, despite the toxicity of the constructs when *E. coli* HER 1025 was
217 transformed with CRISPRi targeting the *cI* gene, transformed colonies were still obtained
218 (Figure 1F). Transformation with a non-targeting CRISPRi yielded two colony sizes, large and
219 small. These two phenotypes were not stable and were rapidly lost, suggesting a transient
220 phenotype linked to the electroporation. Transformation with a *cI*-targeting CRISPRi also
221 yielded two colony types, some were large white colonies and others were very small and
222 translucid.

To determine why some colonies survived transformation with a *cI*-targeting CRISPRi, a PCR test was performed on 10 colonies from each phenotype to investigate i) whether the spacer that targets *cI* had been deleted or ii) whether the transformed cells were free of prophage λ. We observed that 8/10 of the small translucid colonies were cured of prophage λ, whereas 10/10 of the big white colonies were still lysogenic and one of them had lost the spacer on the plasmid. In addition, it should be noted that in the 8 out of the 10 small translucid colonies in which λ was removed, 2 of them were still resistant to λ re-infection. Of note, the bacteria were transformed after growth in TSB medium, which contains glucose. This is significant because glucose is responsible for the downregulation of LamB, the receptor for prophage λ,[43] suggesting that growth in TSB may have provided partial protection against λ reinfection in the λ-free *E. coli*.[44] The presence of transformants that still maintain the prophage and the *cI*-targeting spacer could be explained by the existence of different mutations abolishing the activity of the CRISPRi.

To compare the efficiency of CRISPRi prophage induction and curation with other methods, we performed the induction of λ with 1 µg/mL of mitomycin C. *E. coli* HER 1025 was electroporated with water, and after one hour of recovery, Mitomycin C was added to the medium. After 2h of incubation, bacteria were plated and 50 µL of the induced cultures were added to 10 mL of TSB for an overnight incubation. The day after, phages were titered from the filtered supernatant and prophage deletion was screened by PCR on the bacterial colonies that survived the induction. When the induction was triggered by mitomycin C the titer of λ was $3.5 \pm 4.8 \times 10^9$ PFU/mL while the titer was $7.5 \pm 5 \times 10^8$ PFU/mL when induced by the CRISPRi targeting *cI-1* (Table S4). Thus, λ induction with mitomycin C was slightly more efficient than by the CRISPRi in the tested condition. On the other hand, the curing of λ was significantly higher with the CRISPRi induction as 8 out the 10 colonies tested were cured of λ compared to only 3 out of 49 colonies tested when mitomycin C was used (Table S1).

**Prophage induction using CRISPRi efficiently induces the non-inducible prophage P2 and generates prophage-free derivatives.**

To demonstrate that CRISPRi is a valuable tool for prophage research, the CRISPRi system was also tested on *E. coli*-infecting phage P2. This prophage has been known for decades for being non-inducible, despite numerous trials.[32,33] The genetic organisation of the immunity region of P2 is illustrated in Figure 2A. The P2 repressor is known as C and its activator is called Cox. Both of these proteins are able to bind to the operator sequences that overlap the -35/-10 boxes of each other's promoter, C is binding the operator sequence that overlap *cox*'s promoter and Cox is binding operator regions on the promoter of *C*. Furthermore, the C and Cox transcripts overlap and are transcribed in opposite direction. The transcription of one of the gene then impedes the transcription of the other gene.[31]

We constructed the spacer-containing pCRISPRathBrick targeting the promoter sequences (the -10 and -35 boxes) of the repressor *C* gene (Figure 2A). A spacer targeting a sequence upstream of the L-tail gene, coding for the capsid completion/stabilization protein, was used as a control. This latter gene is not transcribed during lysogeny and has no role in its maintenance. Plasmids were transformed into a lysogenic strain of *E. coli* (C-2322) containing phage P2.[45] Surprisingly, no significant defects in the transformation efficiency were observed with any of the tested conditions (Figure 2B). Unexpectedly, when P2 lysogens were transformed using the C-targeting CRISPRi plasmid, the resulting colony were significantly smaller (Figure 2D), suggesting a growth defect as a consequence of prophage induction and host cells lysis. This effect was not observed for the other constructs. However, the titer of phage P2 in the supernatant of an overnight culture containing the C-CRISPRi plasmid exhibited a 4-log increase when compared to the titers with the two other conditions (nt-CRISPRi and L-CRISPRi) (Figure 2C). These observations confirmed that P2 was efficiently induced and functional virions were produced. Similar to the λ analyses above, the supernatants

were spotted on the lysogenic strain and no lysis plaques were observed, ruling out the possibility of an ultravirulent P2 mutant contributing to the increased phage titer.

We also investigated the surviving colonies after transformation with the C-CRISPRi plasmid. We first looked for the absence of P2 by PCR. Out of the 20 colonies tested, only one was cured of P2. This clone was also resistant to P2 re-infection, suggesting that re-infection was problematic for the survival of P2-cured bacteria during this experiment. As observed with λ, survival of lysogenic transformants with the C-CRISPRi plasmid was intriguing. We hypothesized that their survival can be due to mutations that will lead to non-functional induction. It should be also noted that even if the spacer C is targeting to *C* promoter sequence, it is also overlapping the transcript of *Cox*. In this case, even if the repression of P2 is removed, activation of the lytic cycle will depend on the transcription of *Cox* and its possible that the phage stay in a stalled process, where it cannot accomplish its lytic or lysogenic cycle. This could explain the lower P2 titer when induced with CRISPRi as compared to λ. In sum, we successfully induced phage P2 and were able to cure the host bacteria of this prophage.

**Discussion**

The study of prophage biology can be very challenging due to the low levels of prophage induction.[32] The persistence of a prophage in its bacterial host genome usually relies on the sole expression of the repressor protein and its stability in the cytoplasm. Induction occurs when a cellular component either removes or sequesters this repressor. In this study, we demonstrate that the use of CRISPRi can be used to silence the expression of a prophage repressor, thereby triggering prophage induction. The CRISPRi system was tested on two well-studied prophages that infect *E. coli*, namely λ and P2. We demonstrated that these two prophages were induced, leading to new virions when CRISPRi targeted their repressor genes. This was particularly interesting for phage P2, which was previously documented as being non-inducible.[32,33]

Interestingly, remnant prophages, prophage-like elements and phage-inducible chromosomal islands also maintained a dormant state using similar mechanisms, suggesting that may also be induced using CRISPRi.[46–48]

Another interesting observation was the relative ease of isolating prophage-cured derivatives among colonies that survived the induction. The substantial proportion of prophage-free bacterial cells was probably due to the strong selection pressure applied by prophage induction. It is likely that the use of the CRISPRi system led to more prophage-free bacteria or alternatively, selected for few bacteria that were already free of the prophage among the cell population. Other techniques have been developed to specifically remove prophages from bacterial genomes, including the induction of prophages,[11,49] the overexpression of integrase or overexpression of excisionase,[10] and triggering recombination with or without Cas9.[10,20–22,50] The advantage of the approach described here are, in addition to its high efficiency, that the induction happens in a single step as well as without the use of mutagenic chemicals.

The promising use of CRISPRi for prophage induction (called hereafter, CRISPRpi) is contingent upon two elements: the development of dedicated tools and the knowledge of a prophage's genetic regulation to maintain lysogeny such as the prophage repressor or its promoter. An interesting feature of the pCRISPathBrick plasmid is the possibility of multiplexing through several spacers targeting different genes at the same time. One could argue that the high frequency of prophage-free bacteria may have been helped by the absence of addiction modules (*e.g.* toxin-antitoxin) in the λ and P2 but can be found sometimes in phage genomes.[51,52] If present, these addiction modules (Figure 3A) could also be targeted by a spacer to increase the frequency of prophage-free bacteria. Another approach could be to target the replication protein, which would block phage genome replication. Silencing these different genes may increase the number of prophage-free bacteria.

Previous studies that used thermoinducible mutants of λ and a switch of temperature as an inducer, exhibited a rate of phage-cured bacteria of around 0.1%.[49] In our experiments, the proportion of prophage cured bacteria was significantly higher with CRISPRpi than with the addition of mitomycin C, which is a well-known mutagenic agent.[53,54] CRISPRpi opens new opportunities to cleanly create prophage-free strains strains, which could then be used as replicative host to amplify various phages. An additional application of CRISPRpi could be the study of the temperate phage biology. If a phage repressor is rapidly targeted at the beginning of the infection, it should drive the infection mainly toward the lytic cycle (Figure 3B). On the other hand, targeting the phage lytic activator (*cro* gene for λ) should send the phages into the lysogenic cycle (Figure 3C). In summary, CRISPRpi should be a very valuable approach for various prophage studies.

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## Legends of Figures

**Figure 1: CRISPRi enables the artificial induction of phage λ.** A) General regulation of the maintenance of prophage in lysogeny. B) General principle of CRISPRi for prophage induction. C) Genetic organisation of prophage λ lysogenic module. Spacers used to target phage λ genome are indicated by blue and green arrows. D) Transformation efficiency of *E. coli* HER 1025 with CRISPRi targeting different regions of the prophage. Efficiency was determined by counting the number of transformants from each condition after transformation with 100 ng of plasmid (mean ± standard deviation, n = 3). E) Titers of phage λ in the supernatant of the lysogenic strain HER 1025 transformed with different CRISPRi constructs after overnight incubation. Titers were determined using plaque assays (mean ± standard deviation, n = 3). F) Morphology of surviving colonies from transformation with different CRISPRi constructs after overnight incubation at 37°C.

**Figure 2: CRISPRi enables the induction of the typically non-inducible prophage P2.** A) Genetic organisation of P2's lysogenic module. Spacers used to target phage P2 genome are indicated by blue and green arrows. B) Transformation efficiency of *E. coli* C-2322 with CRISPRi targeting different regions of the prophage. Efficiency was determined by counting the number of transformants from each condition after transformation with 100 ng of plasmid (mean ± standard deviation, n = 3). C) Titers of phage P2 in the supernatant of the lysogenic strain C-2322 transformed with different CRISPRi constructs after overnight incubation. Titers were determined using plaque assays (mean ± standard deviation, n = 3). D) Morphology of surviving colonies from transformation with different CRISPRi constructs after overnight incubation at 37°C.

**Figure 3: CRISPRpi as a tool for prophage studies.** A) Illustration of possible improvements to increase the proportion of bacteria that are cured of the prophage after its induction by CRISPRi. Targeting the phage replication protein would likely block phage replication. Also, targeting the toxin from a potential toxin-antitoxin module in the prophage genome will avoid bacterial mortality caused by the prophage. B) Manipulation of temperate phage behaviour. Infecting a sensitive cell carrying a CRIPSRi that targets the phage repressor should lead to the lytic cycle. However, targeting the phage's main activator would likely force infecting phages toward lysogeny.

## Supplementary Materials

Table S1: List of bacterial strains used in this study

| Strain | Relevant characteristics | Source |
|---|---|---|
| NEB5-alpha | Derivative of DH5α | New England Biolabs |
| HER 1025 | K12 C600 (λ+) | Félix d'Hérelle Reference Center for Bacterial Viruses |
| HER 1037 | K12S (Indicator strain) | Félix d'Hérelle Reference Center for Bacterial Viruses |
| C-8 | Indicator strain for P2 | [45] |
| C-2322 | Lysogenic strain for P2 | [45] |
| JC163 | NEB5-alpha + pJC50 | This study |
| JC165 | NEB5-alpha + pJC51 | This study |
| JC260 | NEB5-alpha + pJC52 | This study |
| JC76 | NEB5-alpha + pJC53 | This study |
| JC79 | NEB5-alpha + pJC54 | This study |
| JC81-100 | C-2322 + pJC53 | This study |
| JC121-140 | HER 1025 + pJC51 | This study |
| JC222 | NEB5-alpha + pJC55 | This study |

532 Table S2: List of plasmids used in this study

| Plasmid name | Relevant characteristics | Source |
|---|---|---|
| pCRISPathBrick | Plasmid carrying CRISPRi system in *E. coli* $cm^R/Tet^R$, constitutive expression | [38] |
| pFD116 | Plasmid carrying CRISPRi system in *E. coli* $Spec^R$, aTC inducible expression | [37] |
| pJC50 | pCRISPathBrick targeting λ*cI* promoter | This study |
| pJC51 | pCRISPathBrick targeting λ*cI* gene | This study |
| pJC52 | pCRISPathBrick targeting λ*ea47* promoter | This study |
| pJC53 | pCRISPathBrick targeting P2*c* gene promoter | This study |
| pJC54 | pCRISPathBrick targeting P2 *L-tail* gene promoter | This study |
| pJC55 | pFD116 targeting λ*cI* promoter | This study |

533

534 Table S3: List of primers used in this study

| Primer name | Oligonucleotide sequences (5'-3') |
|---|---|
| JC32 / cI KO1 F | AAACACACGCACGGTGTTAGATATTTATCCCTTGGTTTT AGAGCTATGCTGTTTTGAATGGTCCCA |
| JC33 / cI KO1 R | GTTTTGGGACCATTCAAAACAGCATAGCTCTAAAACCAA GGGATAAATATCTAACACCGTGCGTGT |
| JC34 / cI KO2 F | AAACAAAAGAAACCATTAACACAAGAGCAGCTTGGTTTT AGAGCTATGCTGTTTTGAATGGTCCCA |
| JC35 / cI KO2 R | GTTTTGGGACCATTCAAAACAGCATAGCTCTAAAACCAA GCTGCTCTTGTGTTAATGGTTTCTTTT |
| JC36 / ea47 KO F | AAACTATCAGCATCTAGCATGCAACCTATCAAAAGTTTT AGAGCTATGCTGTTTTGAATGGTCCCA |
| JC37 / ea47 KO R | GTTTTGGGACCATTCAAAACAGCATAGCTCTAAAACTTT TGATAGGTTGCATGCTAGATGCTGATA |
| JC38 / Spacer ctrl F | CTTTTCAAGACTGAAGTCTAGC |
| JC39 / Spacer ctrl R | GAGTCCTATGAGCTTCCGAG |
| JC70 / P2 Cprom-1 KO F | AAACGGCATTATAAGACATTAAACGCAATTCATGGTTTT AGAGCTATGCTGTTTTGAATGGTCCCA |
| JC71 / P2 Cprom-1 KO R | GTTTTGGGACCATTCAAAACAGCATAGCTCTAAAACCAT GAATTGCGTTTAATGTCTTATAATGCC |
| JC74 / P2-L KO F | AAACTGCACCGGCGTCCACCGCCCGACTTTTCAGGTTTT AGAGCTATGCTGTTTTGAATGGTCCCA |
| JC75 / P2-L KO R | GTTTTGGGACCATTCAAAACAGCATAGCTCTAAAACCTG AAAAGTCGGGCGGTGGACGCCGGTGCA |
| JC133/ lambda KO cI F2 | TAGTTGTTAGATATTTATCCCTTG |
| JC110/ lambda KO cI R | AAACCAAGGGATAAATATCTAACA |
| JC 94 / Primer P2 F | CCGCGTGTGACCTAGTATCC |
| JC 95 / Primer P2 R | AGCACTTGACGGCGACAATA |
| JC125 / pFD116 insert F | ACAGTTTCTATGTTTTGACATACAT |
| JC126 / pFD116 insert R | ATGGGTATGGACAGATCTCC |
| Lambda_For | TGATCAGAAGGACGTTGATCGG |
| Lambda_Rev | AGAGATTCTTGGCGGAGAAACC |

535 Table S4: Comparison of prophage induction and curing using CRISPRi or mitomycin C.

536 Titers were determined using plaque assays (mean ± standard deviation, n=3).

| | Induction by CRISPRi | Induction by MMC |
|---|---|---|
| Phage titer (PFU/ml) | $7.5 \times 10^8 \pm 5 \times 10^8$ | $3.5 \times 10^9 \pm 4.8 \times 10^9$ |
| Clones cured from λ | 8/10 | 3/49 |

537

Figure 1

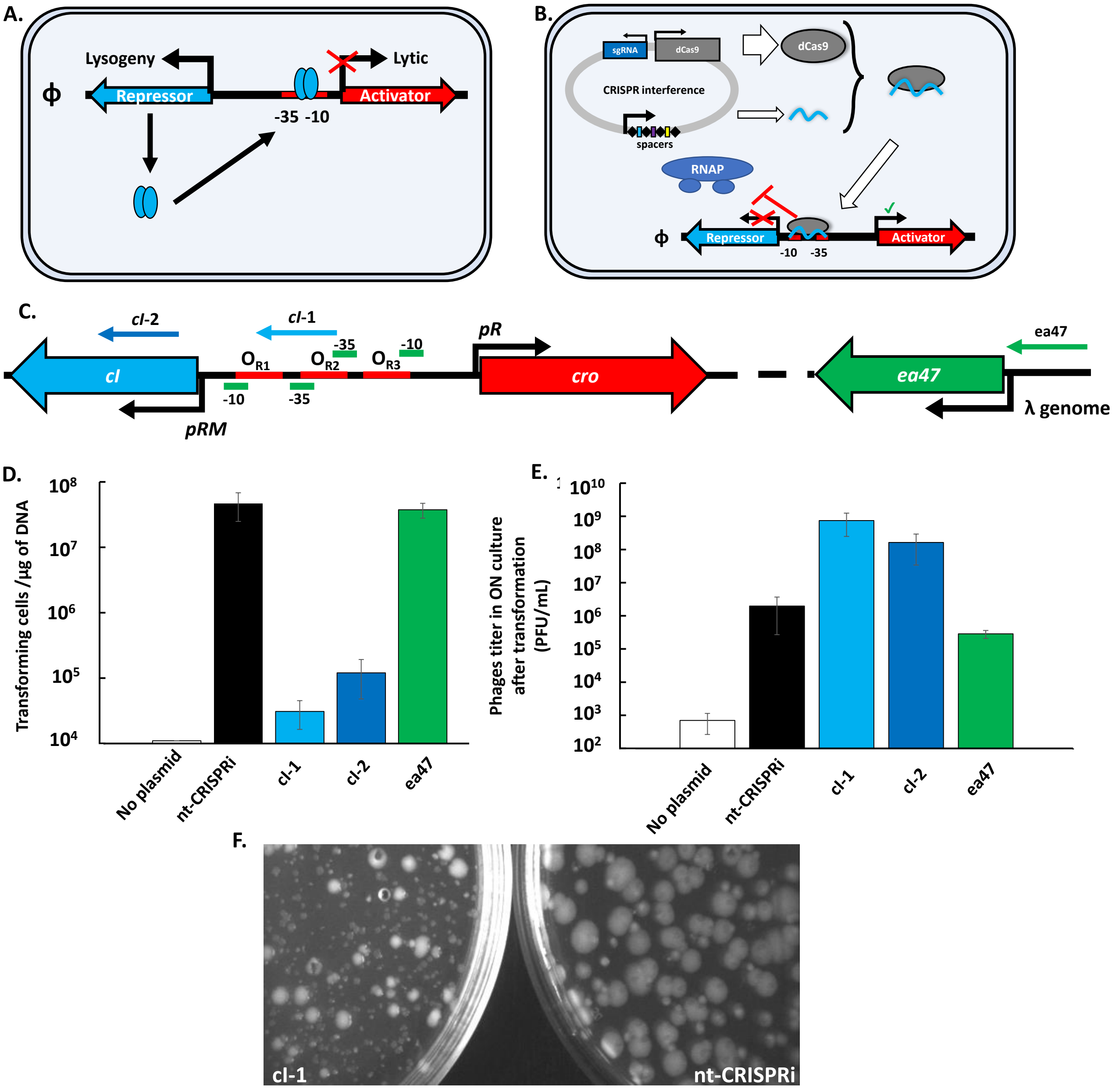
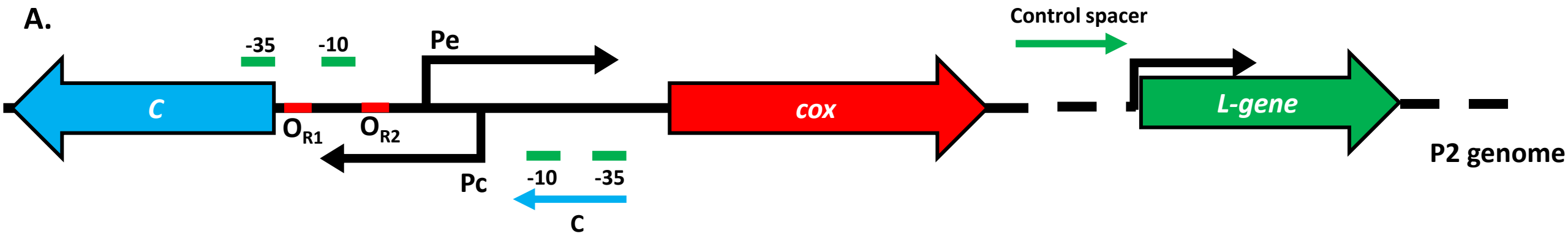
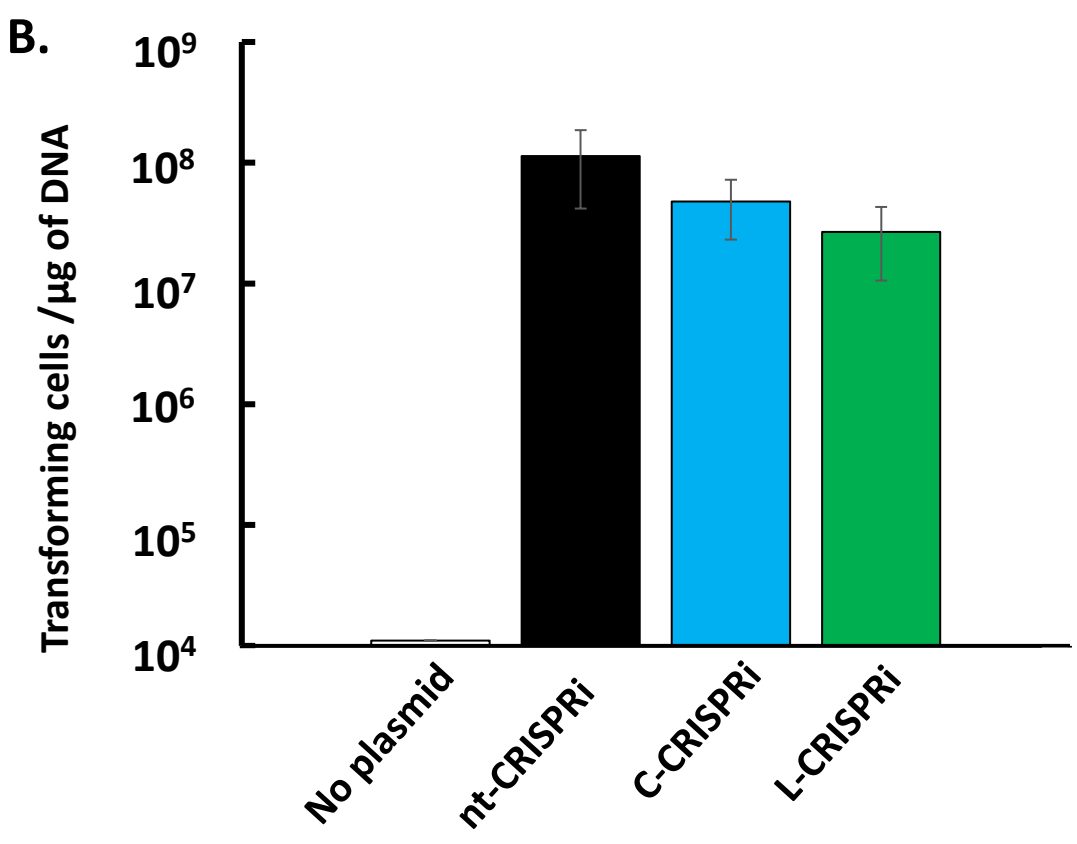
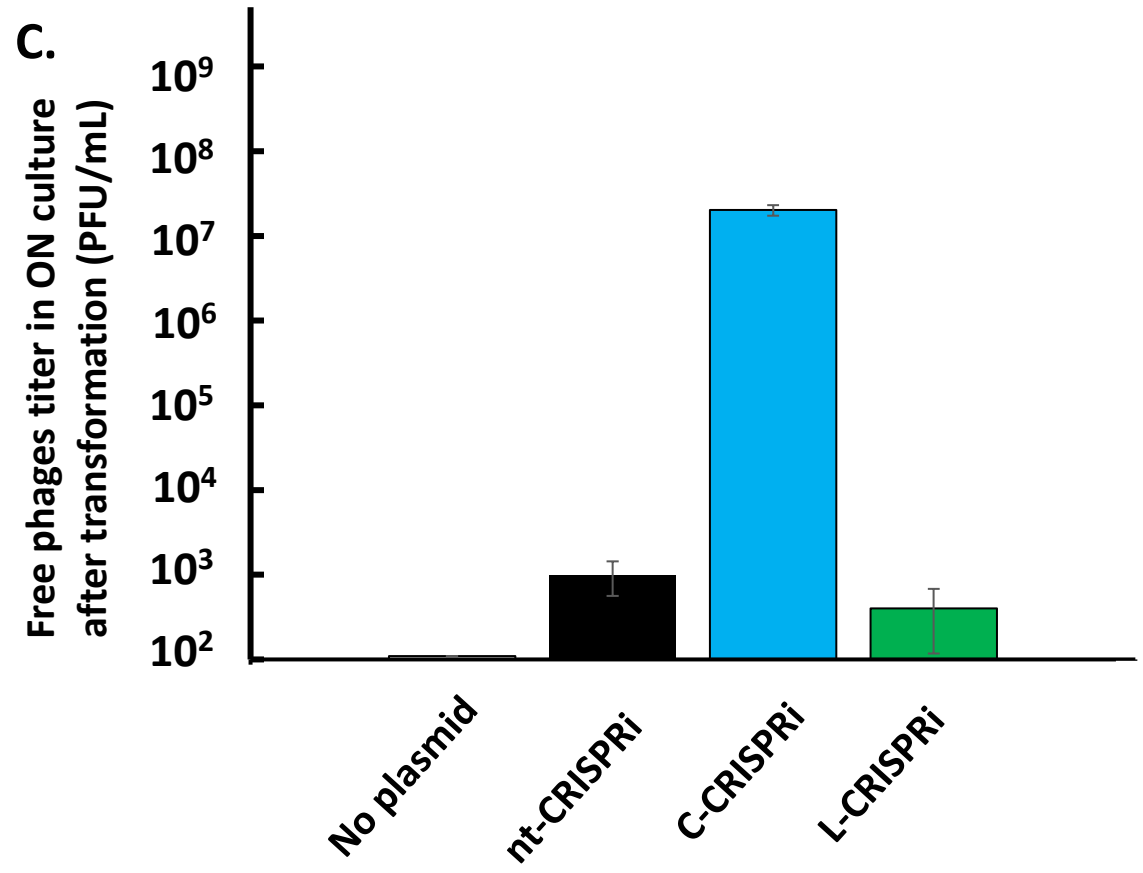
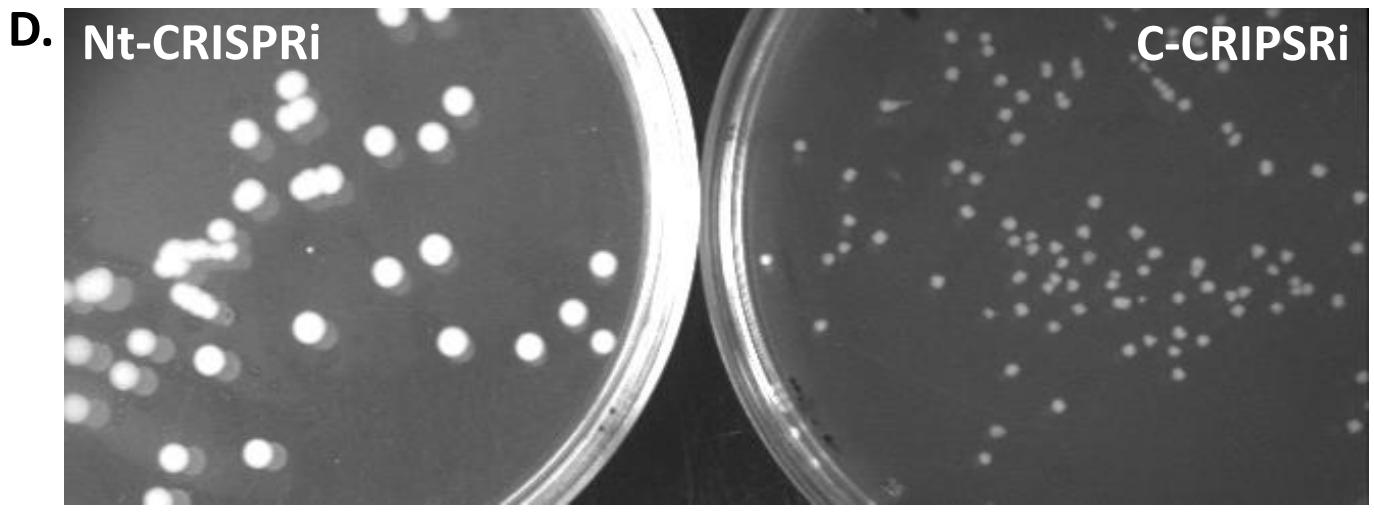

A.
Lysogeny
Lytic
ϕ
Repressor
Activator
-35 -10
B.
sgRNA
dCas9
dCas9
CRISPR interference
spacers
RNAP
ϕ
Repressor
Activator
-10 -35
C.
cI-2
cI-1
pR
-35
-10
O R1
O R2
O R3
cI
cro
-10
-35
ea47
ea47
λ genome
pRM
D.
Transforming cells /µg of DNA
10^8
10^7
10^6
10^5
10^4
No plasmid
nt-CRISPRi
cI-1
cI-2
ea47
E.
Phages titer in ON culture after transformation (PFU/mL)
10^10
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
No plasmid
nt-CRISPRi
cI-1
cI-2
ea47
F.
cI-1
nt-CRISPRi

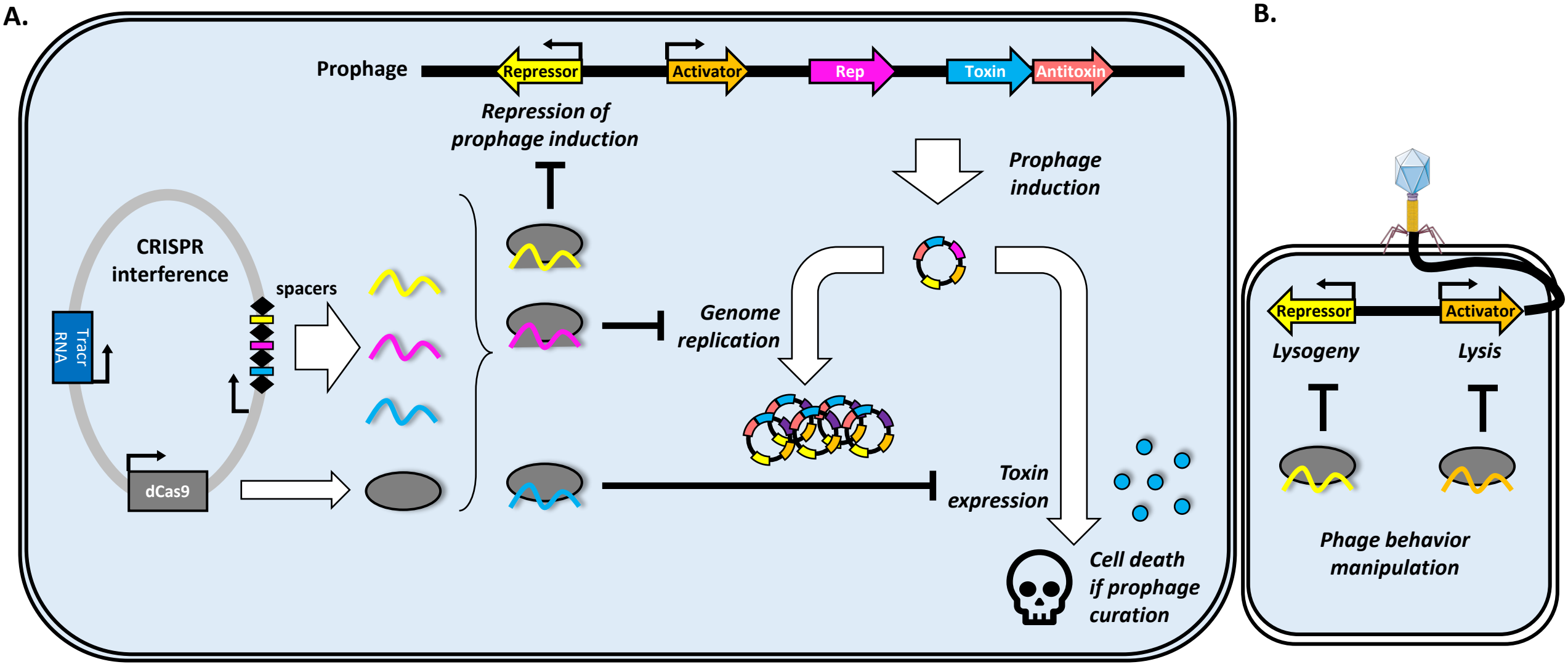
Figure 2


A.
-35
-10
Pe
Control spacer
C
cox
L-gene
$O_{R1}$
$O_{R2}$
Pc
-10
-35
C
P2 genome


B.
Transformation cells /µg of DNA
$10^9$
$10^8$
$10^7$
$10^6$
$10^5$
$10^4$
No plasmid
nt-CRISPRi
C-CRISPRi
L-CRISPRi


C.
Free phages titer in ON culture after transformation (PFU/mL)
$10^9$
$10^8$
$10^7$
$10^6$
$10^5$
$10^4$
$10^3$
$10^2$
No plasmid
nt-CRISPRi
C-CRISPRi
L-CRISPRi


D.
Nt-CRISPRi
C-CRIPSRi


A.
Prophage
Repressor
Activator
Rep
Toxin
Antitoxin
Repression of prophage induction
Prophage induction
CRISPR interference
spacers
Tracr RNA
dCas9
Genome replication
Toxin expression
Cell death if prophage curation
B.
Repressor
Activator
Lysogeny
Lysis
Phage behavior manipulation