

Rapid, expression-free bacteriophage-based specific detection of target bacteria by conditional release of encapsidated reporter molecules

Tamas Feher

fehert@brc.hu

HUN-REN Biological Research Centre of Szeged

Akos Avramucz

HUN-REN Biological Research Centre of Szeged

Joseph Wheatley

Lucidix Biolabs

Richard Amaee

Lucidix Biolabs

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Abstract

Rapid diagnosis of infectious diseases is of paramount importance to prevent or control outbreaks and pandemics. Detection of bacteria is commonly performed using culture-based and molecular detection methods, which cannot address the need for quick, specific and cheap diagnostics. Bacteriophage-based assays rely on the rapidity, specificity and effectiveness of phage-host interactions and can be engineered with fluorescence or luminescence-based reporters. Previous attempts, however, required transcription and translation of reporter genes, leading to long assays and restrictive protocols that work against uptake by untrained users. In this proof-of-concept work, we tested whether the signal generation time could be shortened by detecting the injection of a phage protein, thereby circumventing the need for gene expression altogether. We demonstrate that injection of the N-terminal fragment of the split nanoluciferase protein of *Oplophorus gracilirostris*, fused to the products of genes g6.7 or g14 of phage K1F, is detectable upon injection into an *Escherichia coli* cell harbouring the C-terminal fragment, as early as 5 min 45 s after phage addition. We also demonstrate that the engineered phages generate a signal upon exposure to cognate K1 - but not to non-cognate K5 capsule-enclosed *E. coli* cells - indicating the specificity of our system.

1. Introduction

One of the first lessons learned during the COVID-19 pandemic was the important role of rapid and scalable tests in the early diagnosis and isolation of infected individuals. In parallel, the ever-growing incidence of infections caused by multi-resistant bacterial pathogens[1] underlines the need for rapid tests to identify bacteria also, thereby permitting the early initiation of targeted therapeutic interventions. To detect bacteria, both culture-based and molecular biology-based methods have been widely used. The former is usually time consuming, and requires further downstream analysis carried out by trained personnel. The latter most often rely on polymerase chain reaction (PCR) or antibody-based detection. PCR, especially qPCR, also requires highly skilled staff, expensive infrastructure and in practice also ISO quality certification of the testing lab and its staff. While antibody-based detections are widely applied in bedside test kits, their sensitivity and specificity are often limited. Therefore, there is still a need for a quick, specific and cheap method to detect bacteria in biological samples.

Bacteriophage-based assays to detect bacteria rely on the rapidity, specificity and effectiveness of phage-host interactions. Furthermore, phage can tolerate a high degree of sample impurity (with some phage having first been identified in sewage[2], [3], [4]), which speaks to their potential robustness when deployed in diagnostic kits designed for use by the general public, who lack the training rigour of diagnostic lab staff. Classical phage amplification assays have long been used to identify various bacterial pathogens, and are based on the ability of one or more bacteriophage (phage) strains to replicate (e.g. form plaques) on the pure culture of the target bacterium. Other phage-based methods capture target bacteria via tail-spikes[5], [6], [7] or immobilized phage endolysin components[8], [9], [10]. These latter techniques however usually require further downstream analysis[11], [12]. The advent of genetically engineered phages brought the possibility of assays detecting fluorescent or luminescent

signals that indicate phage infection. These signals are generated by the target cells, after infection by phage, upon expression of the respective phage-borne reporter transgenes injected by the phages. In the current work, we tested whether phage-based signal detection could be shortened by bypassing expression by the direct detection of a ready-made luminescent protein, which undergoes ejection from the phage capsid, in response to the presence of its cognate host. This approach can circumvent the need for phage-borne gene expression as a prerequisite for signal generation – currently the bottleneck in signal detection.

Since we are not aware of signal sequences directing proteins into the capsid of T7-related phages, we chose to fuse our luminescent reporter protein to intracapsid proteins (ICPs) of the chosen phage. The ICPs (and presumably, their fusion derivatives) spontaneously localize to the inside of the capsid[13] which prevents them from premature reactions until their host-triggered ejection. ICPs have been thoroughly studied in phage T7 and we reasoned that their orthologues in the closely related phage K1F would share enough similarity as to allow T7-gained knowledge to be applicable.

The first step of phage infection involves the phage successfully binding to its cognate host. In the next step, the ICP proteins are propelled from the phage particle into the host cytoplasm in a defined sequence: gp6.7 and gp7.3 first exit together[13]. Then, gp14, gp15 and gp16 partially unfold (due to their size exceeding the capsid's aperture) so as to exit the phage nozzle and then spontaneously refold within the host envelope to form the ejectosome: a tubular multi-protein structure. In this process, gp14 forms the outer channel within the host outer membrane, while gp15 and gp16 form a complex to extend the ejectosome tunnel to traverse the periplasmic space and span the inner membrane. Finally, the phage genome is delivered through the ejectosome into the host cytoplasm, where phage propagation commences[14].

A potential luminescent reporter, the nanoluciferase protein from *Oplophorus gracilirostris* can be split into an N and a C terminal fragment (NnLuc and CnLuc, respectively), each of which alone lack activity[15]. Crucially for this application, these enzyme fragments can *spontaneously* recombine in solution, regaining the ability to emit light in the presence of the substrate compound (here Furimazine). We hypothesized that if the smaller N terminal fragment of nanoluciferase (NnLuc) was fused to an ICP (gp6.7 or gp14), and the C terminal nanoluciferase fragment (CnLuc) and Furimazine were provided in the bacterial cell's cytoplasm, the injection of the NnLuc fusion protein into the host cell during infection could be detected due to the resultant luminescence signal. In essence, the NnLuc fragment, necessary to elicit light in the presence of CnLuc and substrate, would be kept sequestered inside the capsid until and unless a cognate host was supplied.

We display here a proof of principle luminescence detection system where two different modified K1F phages are used to detect the presence of an *Escherichia coli* strain protected by a K1 capsule, and expressing the C terminal nanoluciferase fragment (CnLuc). We demonstrate that injection of NnLuc into a bacterial cell harbouring the C terminal fragment (CnLuc) is detectable as early as 5 min 45 s after phage addition, and also in the presence of the transcription inhibitor rifampicin. We further show that

mixing such phages with an *E. coli* strain surrounded by a K5 capsule, i.e. a non-cognate host of the K1F phage does not allow the release of the fusion proteins from the capsid head, and thereby refrains from emitting a luminescent signal.

2. Methods and Materials

2.1. Bacteriophage engineering

Our goal was to engineer phages K1Fe6.7NnLuc and K1Fe14NnLuc. The prior phage carries an extra copy of gene g6.7 in translational fusion with the N terminal part of nanoluciferase (g6.7::NnLuc), integrated into the phage genome just downstream of gene g10b. The latter phage carries an extra copy of gene g14 in translational fusion with the N terminal part of nanoluciferase (g14::NnLuc), integrated into the phage genome just downstream of gene g10b. The donor plasmids pSBC3g6.7::NnLuc and pSBC3g14::NnLuc, respectively, had to be constructed for these two phage engineering purposes. To make these plasmids, we ordered the gene encoding NnLuc as GeneStrands from Eurofins Genomics. NnLuc was designed to carry a flexible linker at its N terminus (see Supplement).

To generate the pSBC3g6.7::NnLuc donor plasmid, we started out with an older donor plasmid, pSBC3[16], which carries a GFP gene flanked by homology arms that direct it to the 3' end of g10b, the minor capsid protein of K1F. First, plasmid pSBC3 was PCR-amplified to exclude GFP and add TGA to 3' end of g10b using primers pSBC3fw and pSBC3rev, using Q5 polymerase and 60 °C annealing to obtain a 4320 bp product. Second, NnLuc was PCR amplified from NnLuc GeneStrands using primers NnLuc67Fw and NnLucRev using Q5 polymerase and 60 °C annealing to obtain a 240 bp product. Third, gene g6.7 was amplified from 100-fold diluted K1F phage lysate using primers g6.7fw and g6.7rev using Q5 polymerase and 60 °C annealing to obtain a 255 bp product. This PCR product includes the promoter of g6.7. The three PCR products were assembled in a Circular Polymerase Extension Cloning (CPEC) reaction[17] using Q5 polymerase and 60 °C annealing. The product was purified using the Gel/PCR DNA Isolation System from Thermo scientific, and electroporated into *E. coli* MDS42, followed by plating on LB + Ampicillin agar plates. Colonies were screened by colony PCR using primers VF2 + NnLucCheckRev, using Taq polymerase and 57 °C annealing to obtain a 609 bp product, and VR2 + NnLucCheckFw, using Taq polymerase and 57 °C annealing to obtain a 350 bp product. Double positive colonies were grown for plasmid preparation. The resulting plasmids were sequence-verified by Sanger sequencing using VF2 and VR2. If both were correct, the plasmid was defined as pSBC3g6.7::NnLuc.

To construct pSBC3g14::NnLuc, the above procedure was repeated, with two changes: i) replacing the g6.7fw and g6.7rev primer pair with g14fw and g14rev, which give a 641 bp PCR product on the K1F genome; ii) replacing NnLuc67Fw with NnLuc14Fw. The NnLuc14Fw + NnLucRev PCR yields a 259 bp product. CPEC, purification, transformation and colony PCR was done identically, as above, but the correct PCR product of VF2 + NnLucCheckRev in this case is 976 bp long.

To construct the K1Fe6.7NnLuc phage, pSBC3g6.7::NnLuc was transformed into *E. coli* EV36. Phage K1F was grown on EV36 + pSBC3g6.7::NnLuc. The resulting phage mix was grown on EV36 + pCas9K1FC2 (described in[16]) for three rounds of phage growth. The third phage lysate was diluted to obtain isolated plaques on a lawn of *E. coli* EV36, and the plaques were screened by plaque PCR using primer pairs g10fw + NnLucCheckRev (930 bp) and NnLucCheckFw + g11rev (481 bp). Both checking PCRs were done using Taq polymerase at 57 °C annealing temperature.

Engineering of phage K1Fe14NnLuc was identical except that the donor plasmid was pSBC3g14::NnLuc. The screening PCR reactions were the same, but the g10fw + NnLucCheckRev PCR yielded a product of 1296 bp, when correct.

Basic processes, such as phage growth, plaque assay, titrating, and phage recovery from plaques were done using established protocols[18].

2.2. Cloning of C-terminal nanoluciferase (CnLuc) gene for bacterial expression

The DNA encoding the C-terminal part of nanoluciferase (CnLuc) protein was ordered from IDT as a GeneBlock. The CnLuc GeneBlock was PCR-amplified using primers CnLucFw and CnLucRev2 using Q5 polymerase, 60 °C annealing. This PCR product was used as a template for a second round of PCR, using primers RBSfw and CnLucRev using Q5 polymerase, 60 °C annealing. This product is the insert fragment (an RBS followed by the CnLuc gene).

The pET24dihyd_his_tetR plasmid was PCR amplified, excluding the 'dihyd' gene while keeping the hexahistidine tag and the stop codon using primers pET24CnLucFw and pET24CnLucRev by Q5 polymerase, 60 °C annealing. This 5208 bp product is the vector fragment.

The insert and the vector fragments were fused using CPEC. The product was purified using the Gel/PCR DNA Isolation System from Thermo scientific, and electroporated into *E. coli* MDS42, followed by plating on LB + Chloramphenicol agar plates. Colonies were screened by colony PCR using primer pairs pET24CheckFW + CnLucRevCheck (585 bp) and pET24CheckRev + CnLucFWcheck (678 bp), both done using Taq polymerase at 57 °C annealing temperature. The resulting plasmids were sequence-verified by Sanger sequencing using pET24CheckFW and pET24CheckRev. If both were correct, the plasmid was defined as pET24CnLuc_his.

In order to gain a plasmid to be used in the target bacterial strain, EV36, an additional plasmid was constructed by PCR using the pZA31CFP_tetR plasmid[19] as a basis. The plasmid was PCR amplified, excluding the 'CFP' gene using primers pZALucFw2 and pZALucRev by Q5 polymerase, 60°C annealing. This 2787 bp product is the vector fragment.

The insert fragment was amplified from the previously constructed, sequence-verified pET24CnLuc_his transferring its RBS, hexahistidine tag, CnLuc gene and stop codon. PCR amplification was carried out

using primers RBSFw and CnLucRev3 by Q5 polymerase, 60 °C annealing.

The insert and the vector fragments were fused using CPEC. The product was purified using the Gel/PCR DNA Isolation System from Thermo scientific, and electroporated into *E. coli* MDS42, followed by plating on LB + Chloramphenicol agar plates. Colonies were screened by colony PCR using primer pairs pZACheckFW + CnLucRevCheck (659 bp) and pZACheckRev + CnLucFWcheck (663 bp), both done using Taq polymerase at 57 °C. The resulting plasmids were sequence-verified by Sanger sequencing using pZACheckFW and pZA31CheckRev. If both were correct, the plasmid was defined as pZA31CnLuc_his_tetR.

2.3. Luminescence measurements

In order to measure luminescence, we mixed 100 µl of various samples with 100 µl of prepared NanoGlo reagent in a white flat bottom 96-well plate obtained from Thermo scientific. Samples were then instantly (< 30s) examined in a Synergy H1 reader using blue luminescence fibres (460 nm). Gain was set to 140. The Gen5 3.11 program was used for instrument control and data collection.

To minimize signal crosstalk between neighbouring wells, samples were laid out in the microplates with one 'isolator well', containing 200 µl of sterile LB medium, separating each group of parallels.

Cellular samples were prepared by growing *E. coli* EV36 cultures (optionally harbouring pZA31CnLuc_his_tetR) induced with 50 ng/mL anhydrotetracycline (aTc), to an OD600 of 0.6 in liquid LB medium, from which they were pipetted into appropriate wells, 35 µl each. Additionally, phage lysates were pipetted into the sample wells, 65 µl each, near-simultaneously with a multichannel pipette, totalling the volume to 100 µl/sample.

For the kinetic measurements, the timer was started when the phages were added to the samples. The NanoGlo reagent was added to the sample wells in a kinetic series followed by a 10 s shaking and luminescence measurement. Each kinetic time point represents the start of the actual luminescence measurement. NanoGlo addition and thus the chemical lysis of the cells occurred approximately 30 s before each kinetic time point.

For the experiments analysing cell-free CnLuc, an overnight culture of BL21(DE3) (optionally harbouring pZA31CnLuc_his_tetR plasmid) was diluted 100-fold, and was grown for 4 hours at 37°C in 5 mL of liquid LB medium + aTc. Cells were pelleted at 10.000 g for 1 min, and resuspended in 0.5 mL lysis buffer (20 mM Na₂HPO₄, 300 mM NaCl, 10 mM imidazole, pH7.4). Cells were lysed by 4 rounds of sonication, lasting 30 s each, with 30 s pauses in between, then centrifuged for 5 min at 10.000 g. The supernatant was collected for testing CnLuc activity.

To analyse heat-treated phages, bacteriophages were incubated at 75°C for 5 minutes. Next, 300 µl of heat-treated or untreated phages were mixed with 75 µl of the sonicated cell supernatant. The mixture was left at room temperature for 10 min and divided into wells, 100 µl each, followed by luminescence measurement.

3. Results

To generate K1Fe6.7::NnLuc phages, which carry an extra copy of gene g6.7 in translational fusion with the N terminal part of nanoLuciferase (NnLuc), we carried out the procedures described in Methods and Materials. In summary, wild type K1F phages were grown on *E. coli* EV36 harbouring the donor plasmid (pSBC3g6.7::NnLuc). To select for recombinant phages, the resulting phage mix was grown on EV36 + pCas9K1FC2, which cleaves the wild type, but not the recombinant phage's genome. Phage K1Fe14::NnLuc was generated in a similar fashion, using the donor plasmid pSBC3g14::NnLuc.

3.1. *In vitro* tests

3.1.1. Testing the activity of NnLuc fusion proteins released from phage heads

The genomes of K1Fe6.7::NnLuc and K1Fe14::NnLuc phages contain an extra copy of the gene g6.7 and g14, respectively, in translational fusion with the NnLuc gene. To test whether these fusion genes (g6.7::NnLuc and g14::NnLuc) are packaged into the phage heads, we released all capsid-enclosed proteins by heat inactivating the recombinant phages. These heat inactivated phages were mixed with the cellular extract of *E. coli* BL21(DE3) harbouring the induced pZA31CnLuc_his_tetR plasmid. The binding of g6.7::NnLuc and g14::NnLuc, potentially released from the phage heads to the CnLuc proteins extracted from the induced producer *E. coli* should give a luminescence signal measurable at 460 nm. Indeed, the luminescence values were significantly above the background levels measured for empty LB medium (Fig. 1).

In a second experiment, the luminescence signals deriving from similar samples (cell extract + phages) were compared to those produced by the control: plasmidless BL21(DE3) extract mixed with heat-treated wild type (wt) K1F phage. Heat treated K1Fe6.7::NnLuc and K1Fe14::NnLuc phages produced luminescence signals that were significantly higher than the control when mixed with the extract of BL21(DE3) + induced pZA31CnLuc_his_tetR (Fig. 2.). No increase in signal was observed when the extract derived from plasmidless cells. Similarly, no increase in luminescence was seen if the BL21(DE3) + induced pZA31CnLuc_his_tetR extract was mixed with heat-inactivated K1F wt phage or intact K1Fe14::NnLuc phage. The only unexpected increase in luminescence was seen when BL21(DE3) + induced pZA31CnLuc_his_tetR extract was mixed with intact K1Fe6.7::NnLuc phage, probably caused by contaminating soluble g6.7::NnLuc. We concluded that fusion proteins g6.7::NnLuc and g14::NnLuc are packaged into K1Fe6.7::NnLuc and K1Fe14::NnLuc phages, respectively, and these recombinant proteins are capable of producing light when liberated and bound to free CnLuc.

3.2. *In vivo* experiments

3.2.1 Endpoint luminescence measurements of phage-bacterial interactions

In these experiments, bacteria expressing the CnLuc gene from a plasmid were challenged with recombinant phages K1Fe6.7::NnLuc and K1Fe14::NnLuc. The negative control was the plasmidless *E. coli* EV36, challenged with the K1F wt phage. The luminescence signals measured ~12 min after the addition of the phages were significantly higher than the negative control in the case of *E. coli* EV36 + pZA31CnLuc_his_tetR when challenged by either recombinant phage (Fig. 3). The luminescence was indistinguishable from the control in the case of *E. coli* Nissle 1917 + pZA31CnLuc_his_tetR challenged with either phage, or plasmidless EV36 challenged with either phage. This is the expected result, for the cognate host (*E. coli* EV36, covered by the K1 capsule and carrying the induced pZA31CnLuc_his_tetR) bind to the phage and allow the injection of the phage capsid content leading to the binding of CnLuc to the injected NnLuc fusion protein, ultimately producing a luminescence signal. (In this case the NnLuc fusion protein derives both from the virion content and from the transcription/translation of the injected phage DNA). Also as expected, the non-cognate host (*E. coli* Nissle 1917, covered by the K5 capsule and carrying the induced pZA31CnLuc_his_tetR) produced no luminescence signal with either recombinant phage, since binding of the phages to it is not possible, disallowing the injection of the capsid content. In the other samples, the N or the C terminal fragment of the split nanoluciferase was missing, explaining the lack of luminescence.

3.2.2 Kinetic luminescence measurements of phage-bacterial interactions

In these experiments, bacteria expressing CnLuc from a plasmid were challenged with recombinant phages K1Fe6.7::NnLuc and K1Fe14::NnLuc. The reaction was stopped by addition of the NanoGlo reagent at regular time intervals, and the resulting luminescence was recorded. We expected the cognate host (*E. coli* EV36, covered by the K1 capsule and carrying the induced pZA31CnLuc_his_tetR) to bind to the phage and allow the injection of the capsid content leading to the binding of cellular CnLuc to the injected NnLuc fusion protein, ultimately producing a luminescence signal. (Before 8 min, the NnLuc fusion protein derives from the injected virion content only. After 8 min, additional NnLuc fusion protein is produced by the transcription/translation of the injected phage DNA[20]). We also expected the non-cognate host (*E. coli* Nissle 1917, covered by the K5 capsule and carrying the induced pZA31CnLuc_his_tetR) to avoid binding of the phage, disallow the injection of the capsid content and therefore produce no luminescence signal. The two negative controls were the cognate host (*E. coli* EV36, plasmidless) challenged with the recombinant phage K1Fe6.7::NnLuc and K1Fe14::NnLuc, respectively. The luminescence signals measured ~7 min 50 sec after the addition of either of the recombinant phages were significantly higher than the negative controls in the case of *E. coli* EV36 + pZA31CnLuc_his_tetR, but were indistinguishable from the controls in the case of *E. coli* Nissle 1917 + pZA31CnLuc_his_tetR (Fig. 4). Since in the T7 bacteriophage family the transcription/translation of late

phage genes starts at 8 min or later[20], the significantly increased luminescence signal measured at 7:50–7:55 mark the functionality of the NnLuc fusion proteins injected from the phage head.

3.2.3 Verifying the source of luminescence

To further confirm that luminescence signals, significantly differing for the cognate and non-cognate hosts, are produced by the injected NnLuc fusion proteins as opposed to being transcription/translation products of the injected phage genome, two experiments were made. The first one repeated the *in vivo* kinetic measurements but included luminescence signal detection at earlier time points, while the second one applied the transcription inhibitor rifampicin to eliminate NnLuc synthesised by the target cell.

Firstly, *E. coli* EV36 + induced pZA31CnLuc_his_tetR or *E. coli* Nissle 1917 + induced pZA31CnLuc_his_tetR was challenged with either K1Fe6.7::NnLuc or K1Fe14NnLuc phages, concentrated to a titre of 5×10^{10} phages/ml. After 5 min 45 s of incubation, NanoGlo buffer was added to lyse the cells, thereby functionally halting protein expression. After a further 10 min incubation, the NanoGlo substrate was added and the luminescence was recorded at 460 nm. As apparent on Fig. 5, the luminescence signal detectable upon K1Fe6.7::NnLuc challenge (part A) or K1Fe14NnLuc challenge (part B) was significantly higher when a cognate host (*E. coli* EV36) was coincident than in the presence of a non-cognate host (*E. coli* Nissle 1917). Since in the T7 bacteriophage family, the transcription/translation of late phage genes (including g6.7 and g14 orthologues) starts at 8 min or later[20], the significant luminescence signal measured at 5:45 provides further evidence for the functionality of the NnLuc fusion proteins injected from the phage head.

In the second experiment, cognate (*E. coli* EV36 + induced pZA31CnLuc_his_tetR) or non-cognate (*E. coli* Nissle 1917 + induced pZA31CnLuc_his_tetR) host cells were challenged with either of the two recombinant phages (K1Fe6.7::NnLuc or K1Fe14::NnLuc). After 4 min of incubation, transcription in the cognate host was stopped by adding 50 µg/ml rifampicin (Fig. 6). Rifampicin treatment had only a minor effect on the luminescence produced by the cognate host at 6 min 30 s: transcription inhibition caused a 26% decrease in the case of K1Fe6.7::NnLuc ($p = .01$) (Fig. 6.A), but there was no significant decrease resulting from transcription inhibition during K1Fe14::NnLuc infection ($p = .089$) (Fig. 6.B). As expected, the luminescence levels plateaued upon transcription inhibition, as opposed to the continuing increase seen for the untreated cognate host.

4. Discussion

The aim of this work was to test whether injection of a protein from inside the capsid head of a phage into the cytoplasm of a cognate bacterium can be directly detected using readily available laboratory hardware. The general strategy was to fuse phage ICPs, destined to enter the target cell, to readily-detectable protein reporter partners. In principle, the reporters could be chromogenic[21] or fluorescent[22] proteins. However, we decided to use the nanoluciferase protein as a fusion partner

because luminescence is claimed to possess a signal-to-noise ratio that is far superior to the two prior reporter categories[23].

Nanoluciferase is an engineered derivative of the luciferase of *Oplophorus gracilirostris*, which offers a small size (19.1 kDa), long activity half-life and a high specific activity[24]. A further refinement to our design was the use of the split, self-assembling form (NnLuc with CnLuc) of nanoluciferase[25] which circumvents two potential problems: background luminescence originating from unattached phages and concerns that larger fusions might disrupt ICP capsid entry/exit.

The main principle in our phage design was to minimize the fitness cost of fusing a reporter protein to an ICP. For this reason, we inserted an additional copy of the gene encoding the ICP::NnLuc engineered fusion, leaving intact the wild-type ICP gene. Our fusion genes were driven by the natural promoter of the ICP, and were inserted in a genomic position of the K1F phage that we found to withstand insertions without an observable genetic instability[16], [26]. To provide some flexibility to our tests and as a first step to determining the general applicability of our approach, we conducted parallel experiments fusing the reporter protein to the two smallest ICPs: g6.7 and g14. Based on the same reasoning, we chose NnLuc, the smaller of the two split fragments to be encoded on the phage genome as the fusion partner.

Our initial, *in vitro* experiments applied heat treatment to release the content of the phage capsids in the presence of His-purified recombinant CnLuc proteins. The emerging luminescence signals detected successfully verified the presence and the functionality of the ICP::NnLuc proteins in the capsid of both recombinant phages (K1Fe6.7::NnLuc and K1Fe14::NnLuc). Next, we carried out *in vivo* experiments that monitor luminescence when CnLuc-expressing *E. coli* cells are challenged with the recombinant phages. A significant increase in luminescence was only observed if the CnLuc-expressing *E. coli* strain was surrounded by the K1 capsule, known to be required for K1F docking. *E. coli* Nissle 1917, expressing CnLuc, but covered by a K5 capsule, did not display a luminescence increase when challenged by either of the recombinant phages. This is a demonstration of the potential selectivity of the test, indicating that phage binding is a prerequisite of NnLuc injection, nLuc assembly, and subsequent luminescence. Most importantly, the detectable luminescence increase was already significant at 5 min 45 s for both recombinant phages when infecting the cognate host. Late genes (including g6.7 and g14) of the T7 phage (and presumably of the closely related K1F) are expressed earliest at 7–8 minutes after infection[27]. This indicates that transcription/translation of the reporter genes was not the origin of their detection. In addition, inhibiting the transcription of the infected host with rifampicin caused only a minor, if any, reduction in the luminescence measured at 6 min 30 s. These findings, to the best of our knowledge, provide the first pieces of evidence that the injection of a protein from the phage into the target cell can be used to detect successful phage infection.

This work acts as a proof of concept for the development of a diagnostic tool detecting bacterial strains against which NnLuc expressing phages are developed. This technology readily lends itself to cheap, lateral flow style diagnostic tests that allow for rapid detection in the field without need for any further device or power. Any diagnostic device would likely substitute a chromogenic reporter for ease of

detection outside of a lab setting. Such a diagnostic would provide all elements required for signal other than the target (host bacteria). Taking the current work as an example, these would be Furimazine (the substrate of nLuc), lyophilised or otherwise dry-stabilised CnLuc and of course the NnLuc-bearing phage. The user would supply the host along with the moisture required to initiate any potential reaction. Potentially, a lysing reagent would also be added to facilitate union of cytosolic NnLuc with the supplied exogenous CnLuc.

The development of a reliable protocol is in progress. One shortcoming of our results is the false positive luminescent signal detected when mixing intact K1Fe6.7::NnLuc with a CnLuc extract. We believe this was caused by soluble NnLuc proteins present in phage lysates, and underlines the importance of using high purity recombinant phages and recombinant CnLuc for detection. Improved purity will likely reduce background luminescence, and increase the signal-to-noise ratio of the system. That ratio may also be improved by deleting the intact copies of the wild-type ICP genes from the genomes of the recombinant phages, thereby potentially elevating NnLuc quantity within each capsid, and consequently increasing the achievable luminescent signal intensities. The fitness costs of phages purely expressing the labelled form of the ICP (g6.7::NnLuc or g14NnLuc) need to be determined, however. If wild-type ICP is found to be obligatory, a compromise may be to leave the wild-type ICP copy but with an attenuated promoter, thereby enriching NnLuc capsid loading while maintaining viability. Signal intensities may also be increased by further concentrating phage suspensions by prolonging standard filter-based purification.

Another approach could be to deploy this novel technology within a standard microfluidic device. Microfluidic lab-on-a-chip designs, compared to their corresponding macroscopic reactions, have been shown to allow shorter reaction times and require less substrate thus lowering the detection limit of the system. In many cases, the limited and strictly defined reaction spaces also permit higher sensitivity of detection, and a further improvement in signal-to-noise ratio. We have displayed the schematic of a single-channel microfluidic detection device elsewhere (Liyanagadera et al., submitted). The real power of microfluidics, however, lies in the possibility of fabricating multichannel devices, allowing the testing of multiple samples against multiple phages using combinatorial matrices. One can envision using multi-step microfluidic diagnostic pipelines, where, for example, the first chip merely classifies the genus of the bacterial cells, the second chip (chosen based on the output of the first chip) provides information on the species, and the third chip (chosen based on the output of the second chip) defines the exact bacterial strain.

With the renaissance of phage therapy, defining the serotype of the pathogenic bacterial strain using such a phage-based system far exceeds the value of mere classification: it provides direct, visually-supported information on the phage sensitivity of the pathogenic isolate. The novel diagnostic phages presented in this work could be used in conjunction with therapeutic phages. By combining those phages that generate positive signals, phage cocktails can be quickly prepared and administered. This could result in the accelerated provisioning of personalized antibacterial therapies to previously unseen

rates in precision medicine from hours to minutes – and in a context where minutes may matter when it comes to saving life and limb.

Declarations

Competing Interests

Conflict of interest statement: The authors intend to pursue commercialisation of breakthrough via Lucidix Biolabs (LB). RA is Founder and Director at LB. JW is a shareholder in LB. TF and ÁA intend to pursue further work with LB towards commercial development.

Author Contribution

ÁA carried out experiments, displayed data. JW formulated of the project, carried out experiments. TF designed the experiments, evaluated results, drafted the manuscript. RA formulated the project, consulted with the team, finalized the manuscript. All authors have read and approved the manuscript.

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Data Availability

The datasets generated and analysed during the current study are available in the figshare.com repository under the following DOI: 10.6084/m9.figshare.27377133

References

1. J. O'Neill, "Antimicrobial Resistance: Tackling a crisis for the health and wealth of nations," *AMR Rev.*, vol. Wellcome Trust, London, UK, 2014.
2. E. Ballesté *et al.*, "Bacteriophages in sewage: abundance, roles, and applications.," *FEMS Microbes*, vol. 3, p. xtac009, 2022, doi: 10.1093/femsmc/xtac009.
3. Y. Elahi, J. Nowroozi, and R. M. N. Fard, "Isolation and characterization of bacteriophages from wastewater sources on *Enterococcus* spp. isolated from clinical samples.," *Iran. J. Microbiol.*, vol. 13, no. 5, pp. 671–677, Oct. 2021, doi: 10.18502/ijm.v13i5.7434.
4. V. Runa, J. Wenk, S. Bengtsson, B. V. Jones, and A. B. Lanham, "Bacteriophages in Biological Wastewater Treatment Systems: Occurrence, Characterization, and Function.," *Front. Microbiol.*, vol. 12, p. 730071, 2021, doi: 10.3389/fmicb.2021.730071.

5. A. Singh, N. Glass, M. Tolba, L. Brovko, M. Griffiths, and S. Evoy, "Immobilization of bacteriophages on gold surfaces for the specific capture of pathogens," *Biosens. Bioelectron.*, vol. 24, no. 12, pp. 3645–3651, Aug. 2009, doi: 10.1016/j.bios.2009.05.028.
6. J. W. Kretzer, M. Schmelcher, and M. J. Loessner, "Ultrasensitive and Fast Diagnostics of Viable *Listeria* Cells by CBD Magnetic Separation Combined with A511::luxAB Detection.," *Viruses*, vol. 10, no. 11, Nov. 2018, doi: 10.3390/v10110626.
7. S. Kunstmann *et al.*, "Bacteriophage Sf6 Tailspike Protein for Detection of *Shigella flexneri* Pathogens.," *Viruses*, vol. 10, no. 8, Aug. 2018, doi: 10.3390/v10080431.
8. H. Zhang, B. A. Buttaro, D. E. Fouts, S. Sanjari, B. S. Evans, and R. H. Stevens, "Bacteriophage ϕ Ef11 ORF28 Endolysin, a Multifunctional Lytic Enzyme with Properties Distinct from All Other Identified *Enterococcus faecalis* Phage Endolysins.," *Appl. Environ. Microbiol.*, vol. 85, no. 13, Jul. 2019, doi: 10.1128/AEM.00555-19.
9. H. Chibli, H. Ghali, S. Park, Y.-A. Peter, and J. L. Nadeau, "Immobilized phage proteins for specific detection of staphylococci.," *The Analyst*, vol. 139, no. 1, pp. 179–186, Jan. 2014, doi: 10.1039/c3an01608k.
10. Y.-J. Choi, S. Kim, and J. Kim, "Development of a Magnetic Bead-Based Method for Specific Detection of *Enterococcus faecalis* Using C-Terminal Domain of ECP3 Phage Endolysin.," *J. Microbiol. Biotechnol.*, vol. 33, no. 7, pp. 964–972, Jul. 2023, doi: 10.4014/jmb.2302.02033.
11. G. Walcher, B. Stessl, M. Wagner, F. Eichenseher, M. J. Loessner, and I. Hein, "Evaluation of paramagnetic beads coated with recombinant *Listeria* phage endolysin-derived cell-wall-binding domain proteins for separation of *Listeria monocytogenes* from raw milk in combination with culture-based and real-time polymerase chain reaction-based quantification.," *Foodborne Pathog. Dis.*, vol. 7, no. 9, pp. 1019–1024, Sep. 2010, doi: 10.1089/fpd.2009.0475.
12. A. C. G. Foddai and I. R. Grant, "A novel one-day phage-based test for rapid detection and enumeration of viable *Mycobacterium avium* subsp. *paratuberculosis* in cows' milk.," *Appl. Microbiol. Biotechnol.*, vol. 104, no. 21, pp. 9399–9412, Nov. 2020, doi: 10.1007/s00253-020-10909-0.
13. I. Molineux, "No syringes please, ejection of phage T7 DNA from the virion is enzyme driven," *Mol. Microbiol.*, vol. 40, no. 1, pp. 1–8, 2001.
14. N. A. Swanson *et al.*, "Expression and purification of phage T7 ejection proteins for cryo-EM analysis," *STAR Protoc.*, vol. 2, no. 4, p. 100960, Dec. 2021, doi: 10.1016/j.xpro.2021.100960.
15. T. J. Nelson, J. Zhao, and C. I. Stains, "Utilizing split-NanoLuc luciferase fragments as luminescent probes for protein solubility in living cells.," *Methods Enzymol.*, vol. 622, pp. 55–66, 2019, doi: 10.1016/bs.mie.2019.02.003.
16. C. Moller-Olsen, S. Ho, R. Shukla, T. Feher, and A. Sagona, "Engineered K1F bacteriophages kill intracellular *Escherichia coli* K1 in human epithelial cells," *Sci. Rep.*, vol. 8, p. 17559, 2018.
17. J. Quan and J. Tian, "Circular polymerase extension cloning of complex gene libraries and pathways.," *PloS One*, vol. 4, no. 7, p. e6441, Jul. 2009, doi: 10.1371/journal.pone.0006441.

18. T. Fehér, I. Karcagi, F. R. Blattner, and G. Pósfai, "Bacteriophage recombineering in the lytic state using the lambda red recombinases," *Microb. Biotechnol.*, vol. 5, no. 4, pp. 466–476, Jul. 2012, doi: 10.1111/j.1751-7915.2011.00292.x.
19. T. Feher, "Competition between Transposable Elements and Mutator Genes in Bacteria," *MBF*, vol. 29, no. 10, pp. 3153–3159, 2012.
20. I. J. Molineux, "The T7 group," in *The bacteriophages*, Calendar, Richard ed., Oxford, UK: Oxford University Press, 2006.
21. K. A. Lukyanov et al., "Natural animal coloration can Be determined by a nonfluorescent green fluorescent protein homolog," *J. Biol. Chem.*, vol. 275, no. 34, pp. 25879–25882, 0 2000, doi: 10.1074/jbc.C000338200.
22. M. V. Matz et al., "Fluorescent proteins from nonbioluminescent Anthozoa species," *Nat. Biotechnol.*, vol. 17, no. 10, pp. 969–973, Oct. 1999, doi: 10.1038/13657.
23. M. Mauri, S. Vecchione, and G. Fritz, "Deconvolution of Luminescence Cross-Talk in High-Throughput Gene Expression Profiling," *ACS Synth. Biol.*, vol. 8, no. 6, pp. 1361–1370, Jun. 2019, doi: 10.1021/acssynbio.9b00032.
24. M. Hall et al., "Engineered Luciferase Reporter from a Deep Sea Shrimp Utilizing a Novel Imidazopyrazinone Substrate," *ACS Chem. Biol.*, vol. 7, no. 11, pp. 1848–1857, 2012.
25. J. Zhao, T. Nelson, Q. Vu, T. Truong, and C. Stains, "Self-Assembling NanoLuc Luciferase Fragments as Probes for Protein Aggregation in Living Cells," *ACS Chem. Biol.*, vol. 11, no. 1, pp. 132–138, 2015.
26. S. B. W. Liyanagedera et al., "SpyPhage: A Cell-Free TXTL Platform for Rapid Engineering of Targeted Phage Therapies," *ACS Synth. Biol.*, vol. 11, no. 10, pp. 3330–3342, Oct. 2022, doi: 10.1021/acssynbio.2c00244.
27. F. W. Studier, "Bacteriophage T7," *Science*, vol. 176, no. 4033, pp. 367–376, 1972, doi: 10.1126/science.176.4033.367.

Figures

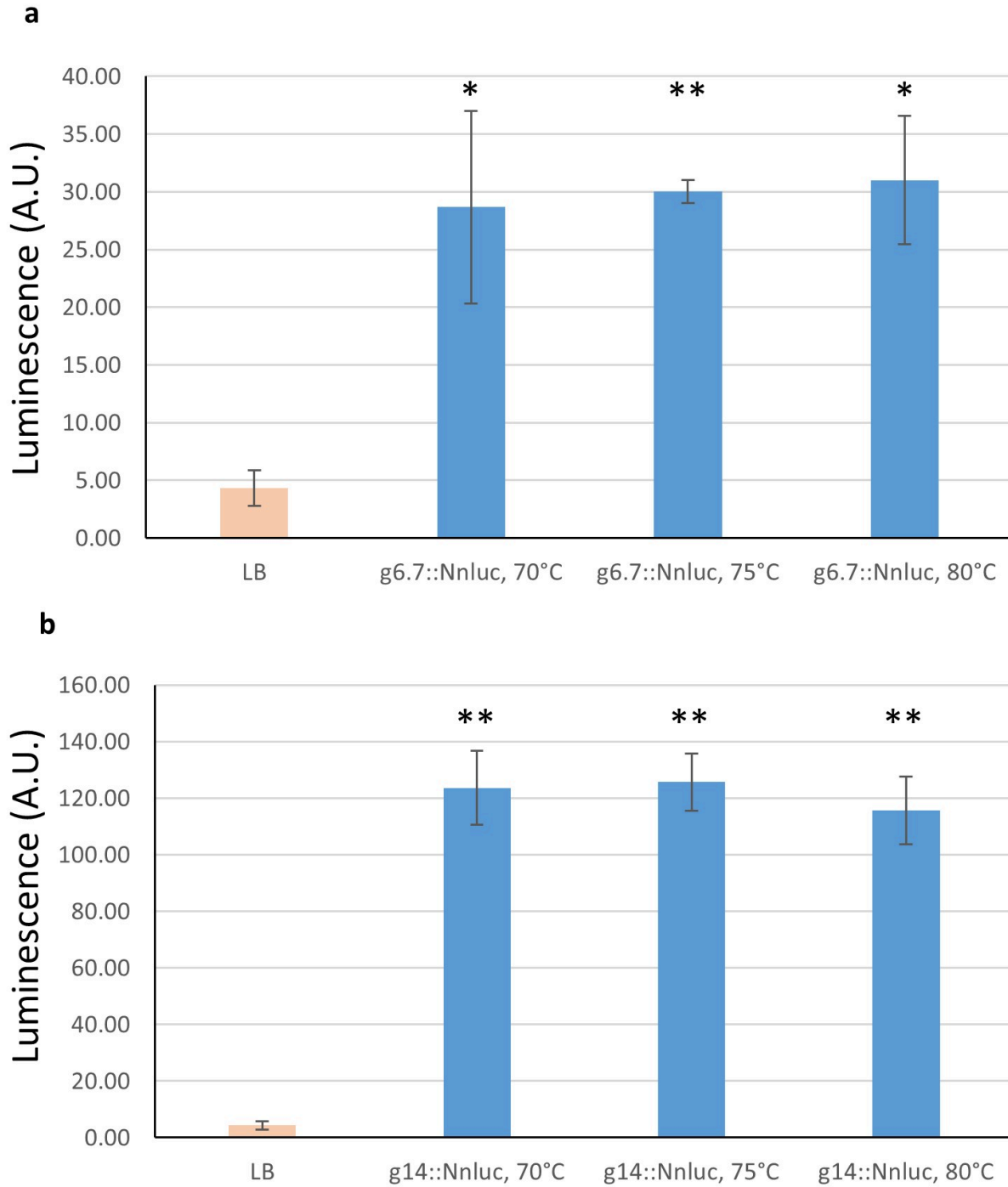


Figure 1

Activity of NnLuc fusion proteins released from the heads of heat-treated phages. K1Fe6.7::NnLuc (a) and K1Fe14::NnLuc (b) phages were heat-treated at the indicated temperatures for 5 min, and mixed with CnLuc extracted from producer cells, as described in the methods. Means of 3 parallel measurements are shown, made on independent samples. Error bars mark standard deviation (SD).

Asterisks mark the results of two-tailed, unpaired t tests comparing each value to the background signal (LB). *: $p < .01$; **: $p < .0001$

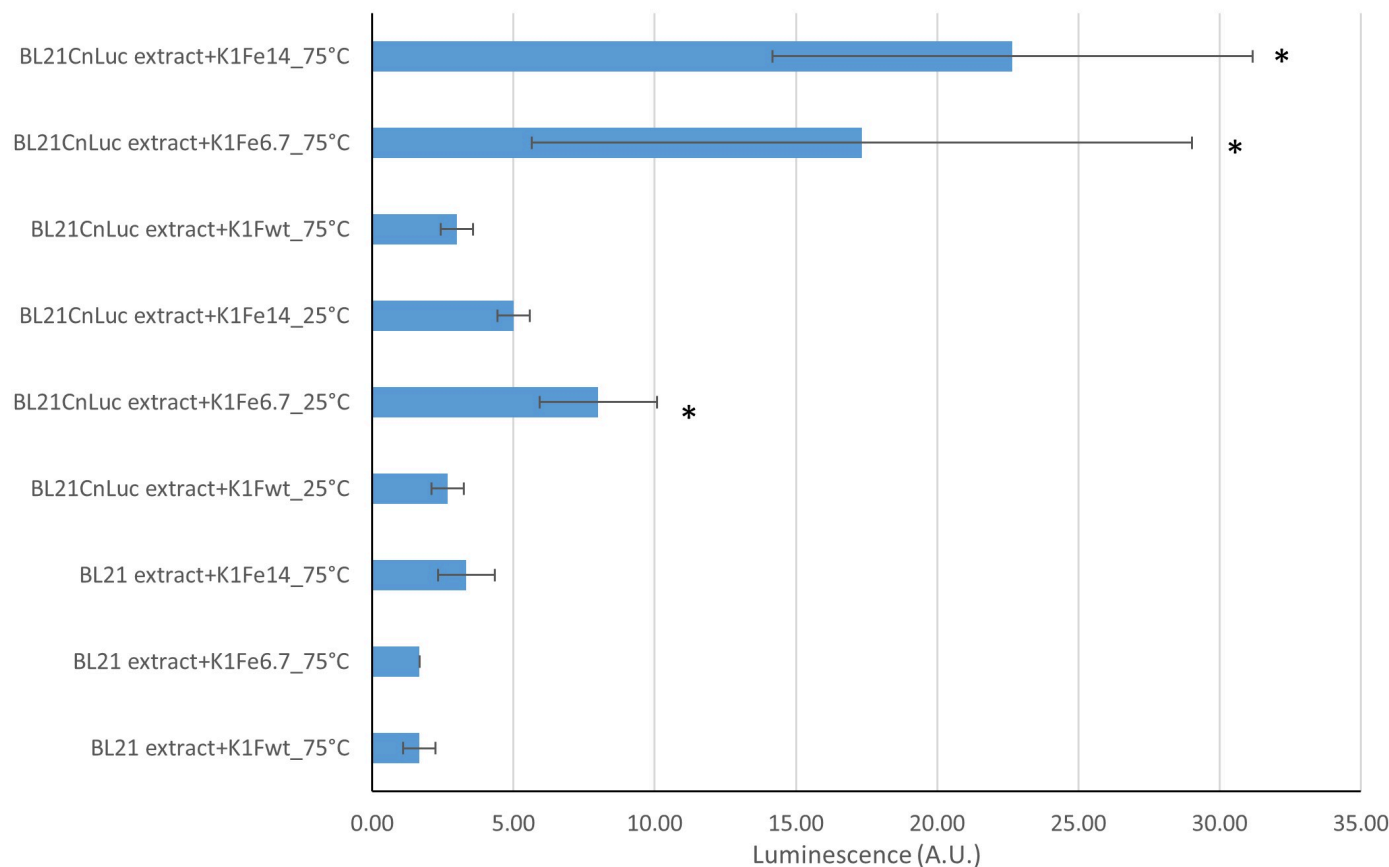


Figure 2

Activity of NnLuc fusion proteins released from the heads of heat-treated phages. BL21CnLuc marks extracts of *E. coli* BL21(DE3) cells harbouring induced pZA31CnLuc_his_tetR plasmid, BL21 marks plasmidless *E. coli* BL21(DE3) cells, K1Fe6.7 marks K1Fe6.7::NnLuc phages and K1Fe14 marks K1Fe14::NnLuc phages. Means of 3 parallel measurements are shown, made on independent samples. Error bars mark SD. Asterisks mark the results of two-tailed, unpaired t tests comparing each value to the control (BL21(DE3) + K1Fwt) signal. *: $p < .05$

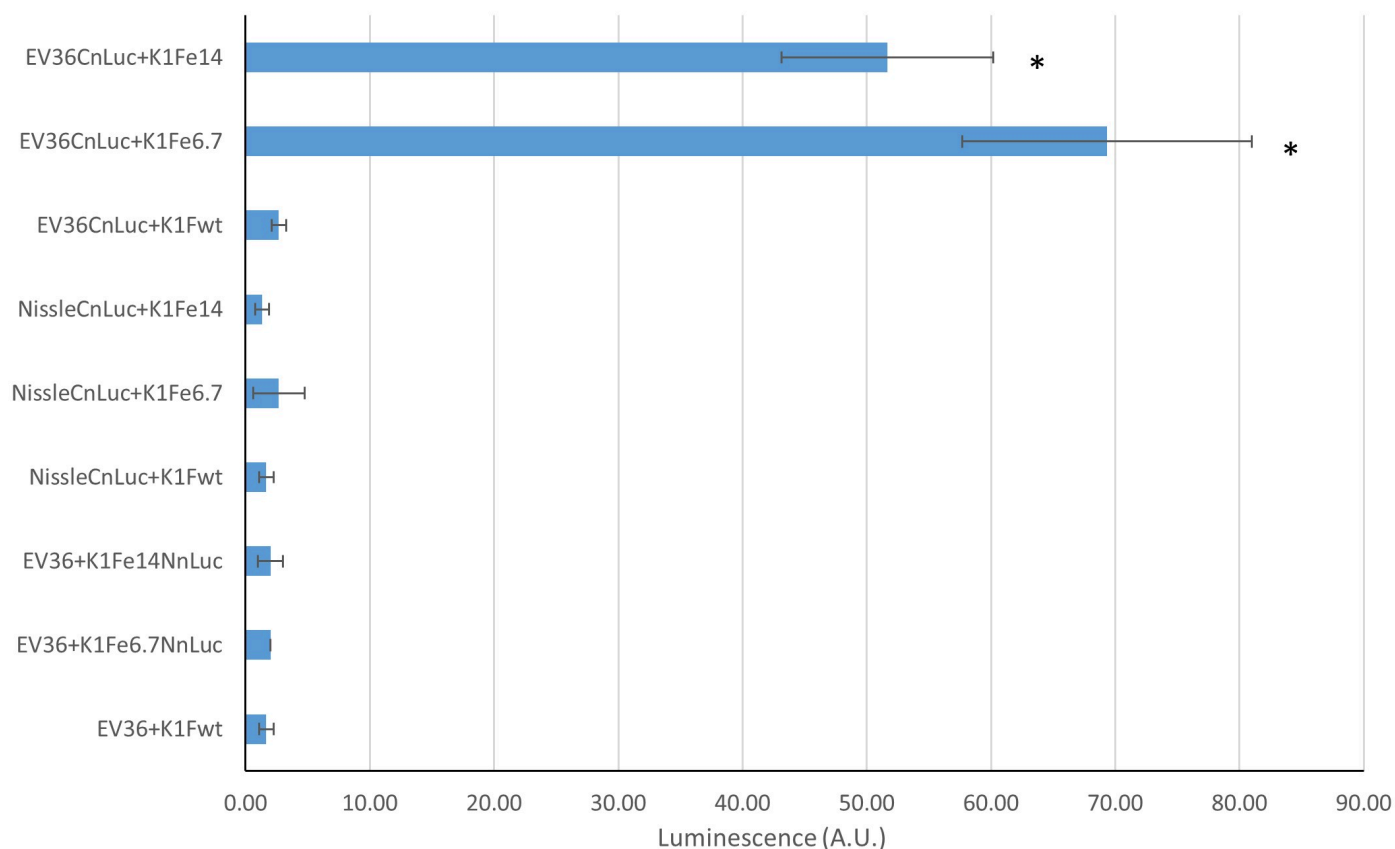


Figure 3

Endpoint luminescence signals produced by various phage-bacterial interactions. EV36CnLuc marks *E. coli* EV36 cells harbouring induced pZA31CnLuc_his_tetR plasmid, EV36 marks plasmidless *E. coli* EV36 cells, NissleCnLuc marks *E. coli* Nissle 1917 cells harbouring induced pZA31CnLuc_his_tetR plasmid. Means of 3 parallel measurements are shown, made on independent samples. Error bars mark SD. Asterisks mark the results of two-tailed, unpaired t tests comparing each value to the negative control (plasmidless *E. coli* EV36 + K1F wt phage). *: $p < .001$

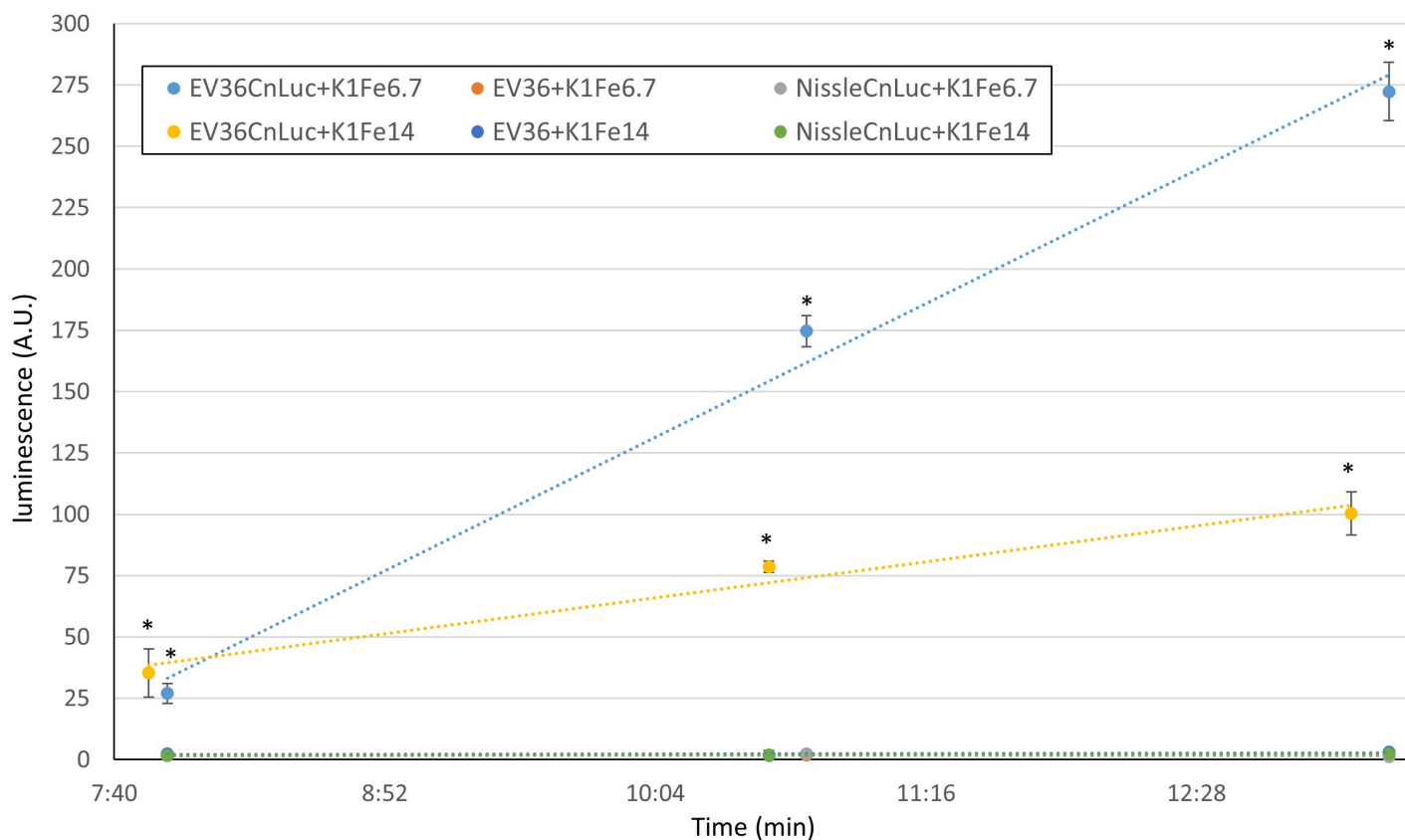


Figure 4

Kinetics of the luminescence signal produced by various phage-bacterial interactions. *E. coli* EV36 + induced pZA31CnLuc_his_tetR (EV36CnLuc) or *E. coli* Nissle 1917 + induced pZA31CnLuc_his_tetR (NissleCnLuc) were challenged with K1Fe6.7::NnLuc or K1Fe14::NnLuc phages. The reaction was stopped by the addition of the NanoGlo reagent at the time indicated, and the luminescence was recorded. Means of 3 parallel measurements are shown, made on independent samples. Error bars mark SD. Asterisks mark the results of two-tailed, unpaired t tests comparing each value to the negative control (plasmidless *E. coli* EV36 + the respective phage). *: $p < 0.01$

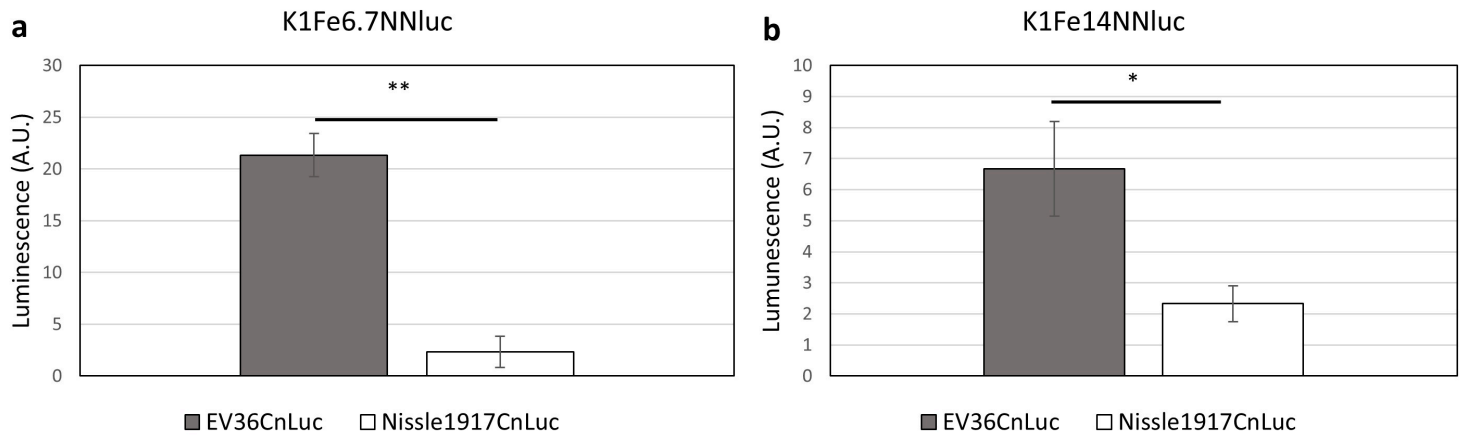


Figure 5

Verification of the functionality of injected NnLuc fusion proteins. Luminescence values were detected 5 min 45 s after challenging *E. coli* EV36 + induced pZA31CnLuc_his_tetR or *E. coli* Nissle 1917 + induced pZA31CnLuc_his_tetR with either phage K1Fe6.7::NnLuc (**A**) or K1Fe14::NnLuc (**B**). Means of 3 parallel measurements are shown, made on independent samples. Error bars mark the SD. Asterisks indicate the results of two-tailed, unpaired t tests comparing the values obtained with the two bacterial strains to each other. *: $p < .05$; **: $p < .001$

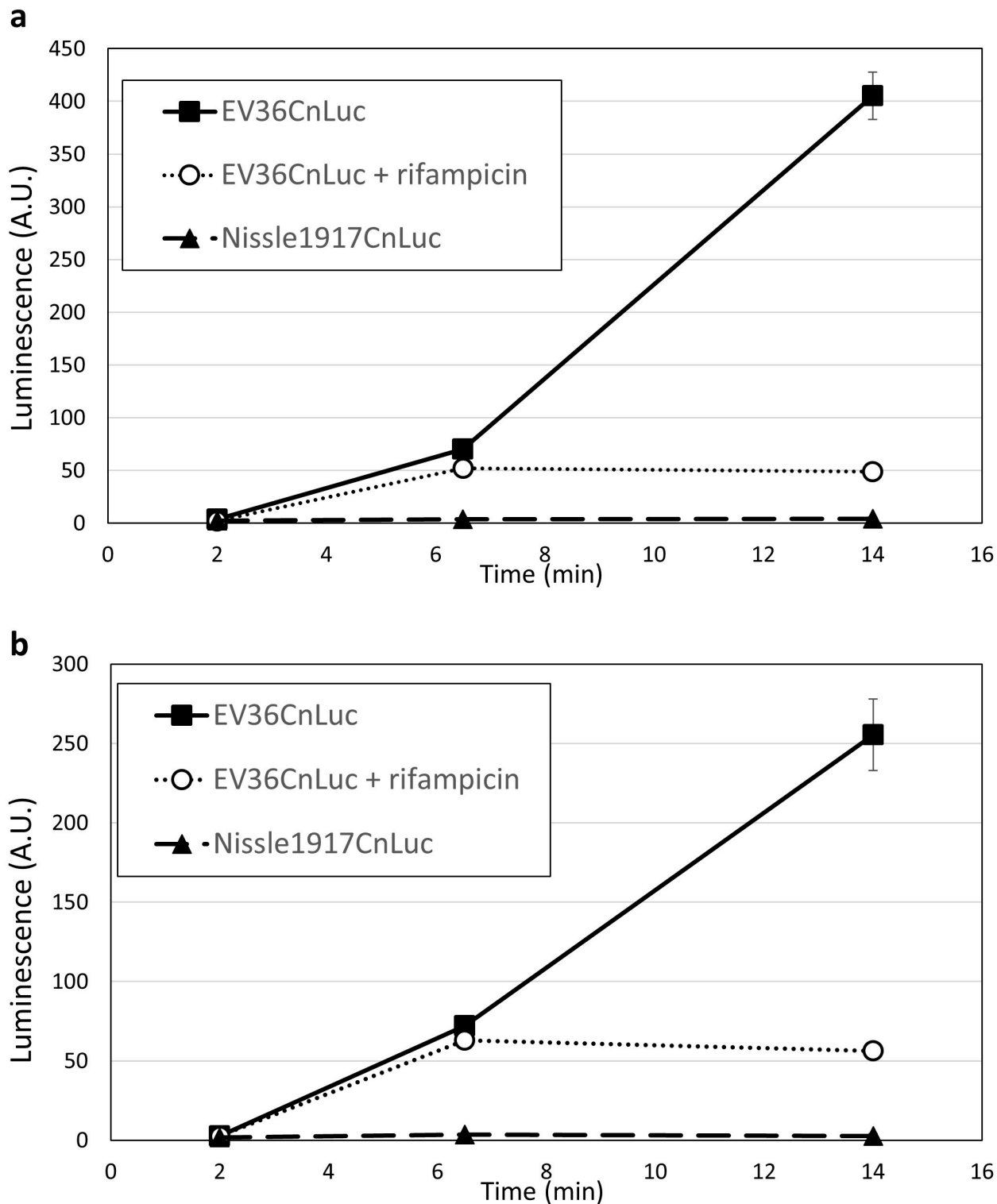


Figure 6

Effect of transcription inhibition on luminescence. Luminescence values were detected at various time points after infecting *E. coli* EV36 + induced pZA31CnLuc_his_tetR (solid line) or *E. coli* Nissle 1917 + induced pZA31CnLuc_his_tetR (dashed line) with either phage K1Fe6.7::NnLuc (a) or K1Fe14::NnLuc (b). Rifampicin was added at 4 min (red arrow) to *E. coli* EV36 + induced pZA31CnLuc_his_tetR (dotted line). Means of 3 parallel measurements are shown, made on independent samples. Error bars mark the SD.

Asterisks indicate the results of two-tailed, unpaired t tests comparing the values to those obtained with *E. coli* Nissle 1917. *: $p \leq .005$; **: $p \leq .001$

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