Analyst

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Journal:	*Analyst*
Manuscript ID	AN-ART-07-2020-001413.R1
Article Type:	Paper
Date Submitted by the Author:	07-Sep-2020
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ARTICLE

Phage-based biosensors: *In vivo* Analysis of Native T4 Phage Promoters to Enhance Reporter Enzyme Expression

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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Phage-based biosensors have shown significant promise in meeting the present needs of the food and agricultural industries due to a combination of sufficient portability, speed, ease of use, sensitivity, and low production cost. Although current phage-based methods do not meet the bacteria detection limit imposed by the EPA, FDA, and USDA, a better understanding of phage genetics can significantly increase their sensitivity as biosensors. In the current study, the signal sensitivity of a T4 phage-based detection system was improved via transcriptional upregulation of the reporter enzyme Nanoluc luciferase (Nluc). An efficient platform to evaluate the promoter activity of reporter T4 phages was developed. The ability to upregulate Nluc within T4 phages was evaluated using 15 native T4 promoters. Data indicates a six-fold increase in reporter enzyme signal from integration of the selected promoters. Collectively, this work demonstrates that fine tuning the expression of reporter enzymes such as Nluc through optimization of transcription can significantly reduce the limits of detection.

Introduction

Bacteriophages (phages) are viruses that are non-infectious towards animals, plants, or humans, instead infecting specific bacteria strains. As such, phages are a promising option to use as a biorecognition factor to detect their host bacteria. Phages, which are ubiquitous in the environment, have evolved to survive broad temperature and pH conditions.[1-3] These inherent properties provide a unique advantage for biosensor applications, resulting in phage-based tools that not only can be used in a variety of sample matrices, but also allow for storage conditions in low-resource settings.[4-6] For industrial applications, phage strains with desirable host ranges can be propagated in bioreactors for large-scale production, which is adaptable for commercialization.[7] In addition, phages have a wide compatibility with frequently utilized biosensor platforms such as PCR,[8] microfluidics,[9] nanoparticle-based,[10, 11] lateral flow,[12, 13] filter-based,[14, 15] surface plasmon resonance,[16, 17] and flow cytometry.[18, 19]

A bacteria detection assay must be sufficiently sensitive to ensure reliability, particularly in circumstances in which the targeted bacteria have a low infectious dose or are present in low concentrations. Many phage-based biosensors utilize "reporter phages" that produce an exogenous protein during phage infection, resulting in a measurable signal for the detection of only viable host bacteria.[20] Luciferase reporters have been utilized as bioreceptors in phage-based biosensing platforms to increase assay sensitivity.[21-24] Due to a relatively low background signal, a bioluminescent signal achieves superior sensitivity compared to other enzyme reporters such as beta-galactosidase, chloramphenicol acetyltransferase, and fluorescent proteins.[25-27] An *Oplophorus gracilirostris* derived luciferase*, NanoLuc luciferase* (*nluc*),[14, 26, 28] is desirable for its small size and ability to produce >150 fold stronger signal compared to traditional luciferases,[29] making it a superior luminescent marker well suited for phage-based biosensors.[30-32] In a previous study, Nluc-phages were successfully used to detect 5 CFU/40 mL of *Escherichia coli* (*E. coli)* O157:H7 in both LB broth (7 hours) and ground beef samples (9 hours).[32]

The rate-limiting factor hindering the practical use of phages for on-site diagnostic tests is the time required to achieve a detectable amount of analyte due to the extended sample preparation process involved.[33] The standard preparation process varies depending on sample origin and complexity, but generally involves steps for increasing the number of target bacteria (enrichment), removing matrix inhibitors, and possibly decreasing sample volume. Additional steps that can be incorporated into the process include (1) filtration steps to selectively capture and concentrate the signal,[14] (2) separation steps utilizing magnetic nanoparticle conjugated phages to capture and concentrate the host bacteria into a smaller sample volume resulting in a stronger signal output,[34] and (3) initial pre-enrichment steps to bring the target bacteria into log phase as well as to help improve the limit of detection of the overall assay by increasing bacterial counts. A combination of these steps has been used to approach the Environmental Protection Agency (EPA) mandated limit of detection of a single CFU of *E. coli* in 100 ml

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

of drinking water.[34, 35] While this strategy increases assay sensitivity, the need to perform pre-enrichment of the sample increases the overall detection time.

The objective of this study is to improve the sensitivity and thus the detection time of phage-based biosensors by manipulating the promoter region that regulates transcription of the reporter enzyme. Here, we present a novel platform to investigate and quantify promoter activity *in vivo* by evaluating 15 different native T4 phage promoters. We employed a lytic *E. coli* infecting T4 phage engineered to produce a Nluc reporter as our baseline. As a model, T4 is one of the best-studied phages,[36] providing ample information on its mechanisms of gene expression to evaluate promoter strength *in vivo.* T4 phages have three classes of promoter: early, middle, and late that initiate transcription by host RNA polymerases at various stages of the infection cycle.[36] T4 phages complete their infection cycle within approximately 30 minutes; therefore, upregulation of the reporter transcript during this allotted time improves signal production and potentially permits earlier detection of luminescent markers in live bacterial cells. This work successfully identifies potent promoters within each class and improves output Nluc signal by six-fold as compared to our previously reported phage biosensors.

Experimental

Bacteria, Phage, and Plasmids

E. coli DH5α was obtained from ATCC #68233 (Manassas, VA USA). pCas9 and pCRISPR were gifts from Luciano Marraffini (Addgene plasmid # 42876 and # 42875; http://n2t.net/addgene:42876; RRID:Addgene_42876; http://n2t.net/addgene:42875; RRID:Addgene_42875). Bacteria overnight cultures (37 °C, 150 rpm, 17 hours) were grown in Luria-Bertani (LB) broth with the appropriate antibiotic (50 µg/mL Kanamycin for pCRISPR, 25 µg/mL Chloramphenicol for pCas9). Wild type T4 phages were obtained from ATCC # 11303-B4 (Manassas, VA USA), propagated, and maintained as described by Bonilla *et al*.[37] T4 phage titers were determined via double layer plaque assay.

Materials and Reagents

All cloning reagents were purchased from New England Biolabs (Ipswich, MA USA). All other reagents were purchased from Thermo Fisher Scientific (Waltham, MA, USA). *E. coli* DH5α electro-competent cells were made in house according to the Untergasser protocol.[38] Nano-Glo luminescent reagent was purchased from Promega (Madison, WI, USA) and prepared immediately before use according to the manufacturer's recommendations. Luminescent signal was monitored via Synergy Neo 2 Hybrid Multimode Reader (Biotek Instruments, Winooski, VT, USA) for 90 minutes at 1-minute intervals paired with microplate orbital shaking also at 1-minute intervals via the Synergy Neo software.

Donor Plasmid Construction

The donor DNA expression cassette containing golden gate cloning sites, native RBS (Ribosomal Binding Site), *nanoLuc luciferase* (*nluc*), *carbohydrate binding module* (*CBM*), synthetic terminator, and regions of homology to *soc* in T4 phages were codon optimized for *E. coli* and synthesized as a gBlock gene fragment (IDT, Coralville, IA, USA) (Figure 1). Gibson Assembly Cloning was employed with NEBuilder HiFi DNA Assembly Master Mix to insert gBlocks into pCRISPR, per manufacturer's instructions. All 15 promoters were individually cloned into the golden gate site using BsaI restriction enzyme. All constructed donor plasmids were screened and confirmed via colony PCR and Sanger sequencing. Sanger sequencing was performed by the Cornell University Institute of Biotechnology, Biotechnology Resource

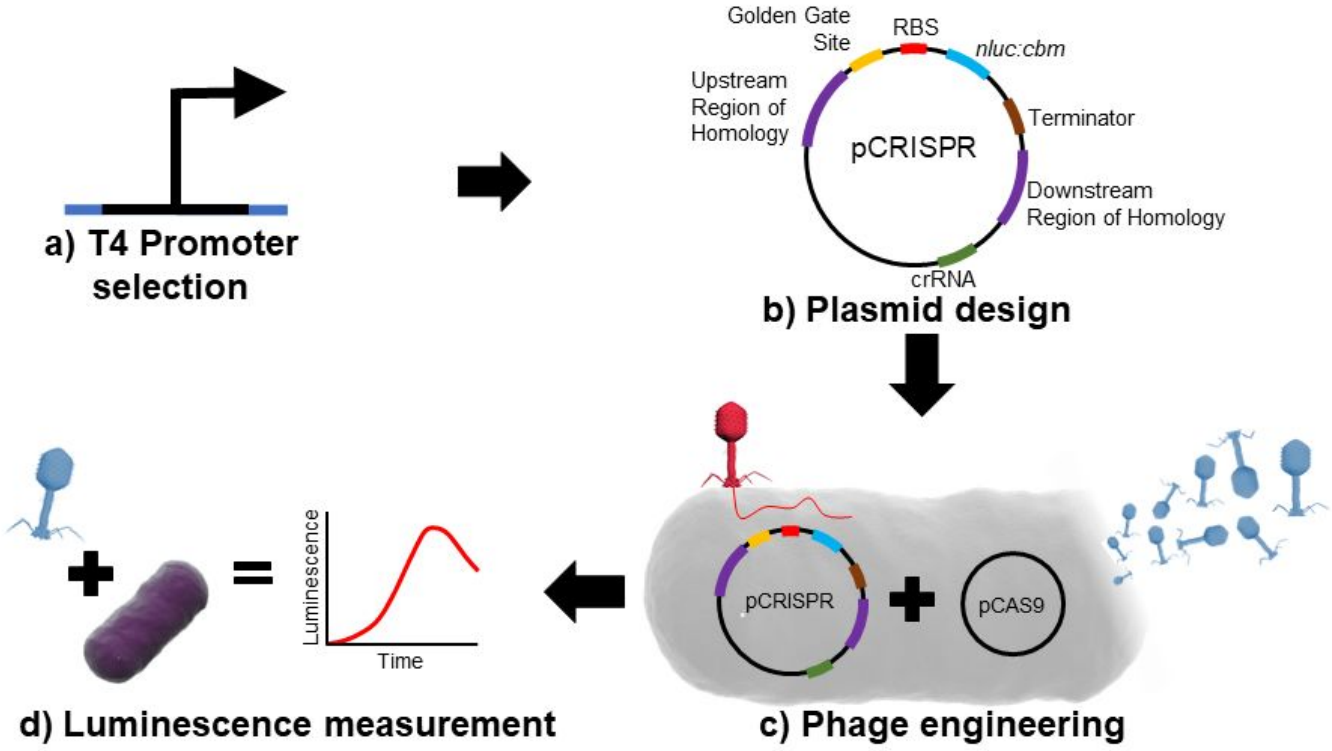

Figure 1: Experimental Overview. a) Native strong promoters were selected from T4 phages and classified into early, middle, and late promoter classes. b) An individual promoter was seamlessly incorporated into the pCRISPR donor plasmid via golden gate cloning. The donor plasmid is tailored for CRISPR/Cas9 mediated engineering and contains a *nluc-cbm* reporter gene flanked by regions of homology to the crRNA recognition sequence. c) CRISPR/Cas9 meditated T4-phage engineering resulting in nluc-phages each contains a newly added T4 phage native promoter. d) The luminescent signal from each nluc phage was measured from time zero to 90 minutes.

Promoter Class	Abbreviated Name	Associated Gene	Selection Criteria		Bioengineered Phage Name
Early	E1	*motB*	Promoter Strength (pKWIII units)	4	NRGp25
	E2	*gp55*		6	NRGp30
	E3	*ipI*		2.7	NRGp31
	E4	DNA ligase		2.6	NRGp32
	E5	*ndd*		3	NRGp33
Middle	M1	*gp34i*	Protein Size (KDa)	140.4	NRGp26
	M2	*gp43*		103.6	NRGp34
	M3	*gp46*		82.9	NRGp35
	M4	*rllA*		63.6	NRGp36
	M5	tRNAscl		N/A	NRGp37
Late	L1	*gp18*	Protein Copy Number	144	NRGp27
	L2	*gp22*		576	NRGp38
	L3	*gp23*		960	NRGp39
	L4	*gp67*		341	NRGp40
	L5	*soc*		870	NRGp41

Table 1: Promoter selection table

Center (Ithaca, NY, USA) using Applied Biosystems Automated 3730xl DNA Analyzers, Big Dye Terminator chemistry, and AmpliTaq FSDNA Polymerase. For nucleotide sequence information, see Fig. S1. Refer to Table S1 for all cloning promoter sequence design.

Recombinant Phages Construction

CRISPR/Cas9 mediated engineering was used to construct recombinant phages as previously reported.[39] The system utilizes pCAS9 to generate the Cas9 endonuclease and pCRISPR to provide the donor DNA sequence and a potent crRNA (TGTGAACGTCAGAATAAAGA) targeting *soc,* the small outer capsid gene. Soc is a nonessential and decorative structural protein with a relatively high copy number of 870/phage particle and therefore is an ideal candidate for genetic modification. Fifteen reporter T4 phages were created, each with an added unique promoter (NRGp25-27 and NRGp30-41) (Table 1) and one reporter control phage without an added promoter sequence (NRGp42). All recombinant phages were confirmed via Sanger and Whole Genome Sequencing (WGS). WGS was performed by Cornell University College of Veterinary Medicine Animal Health Diagnostic Center, Department of Molecular Diagnostics (Ithaca, NY, USA) via the Illumina MiSeq platform and Illumina Basespace Sequence Hub for data acquisition and quality control analysis. All sequencing data were analyzed in Geneious® (Biomatters, Ltd., Auckland, NZ).

Luminescent Assay Procedure

Phage titers (~1.5 × 10^8 PFU/mL), *E. coli* DH5α (~1.5 × 10^7 CFU/mL), and a multiplicity of infection (MOI) of 10 were standardized across all experiments. DH5α, Nano-Glo, and a phage sample (1:2:1 ratio) were added to 96-well white microplate in this order, bringing the final assay volume to 200 µL. All samples were performed in duplicate. The microplate assay was immediately and continuously monitored using the Synergy Neo 2 Hybrid Multimode Reader from time 0 to 90 minutes. The output luminescent signal was quantified as RLU (relative luminescent unit). Assays were repeated with three additional experimental replicates.

Results and discussion

Selection Criteria of T4 Promoters

We sought to identify promoters that will boost the rate of reporter gene transcription initiation and therefore increase the level of mRNA transcripts that are subsequently translated into reporter enzymes. Higher levels of reporter protein will magnify the output signal coming from the phage-based biosensor and thus reduce the pre-enrichment time and total assay time. T4 phages have three classes of promoter: early, middle, and late and each uniquely redirects the host RNA polymerase (RNAP) to successfully transcribe its genome. Fifteen native promoters (Table 1) were selected based on the annotated T4 genome among all three classes of promoter.[36]

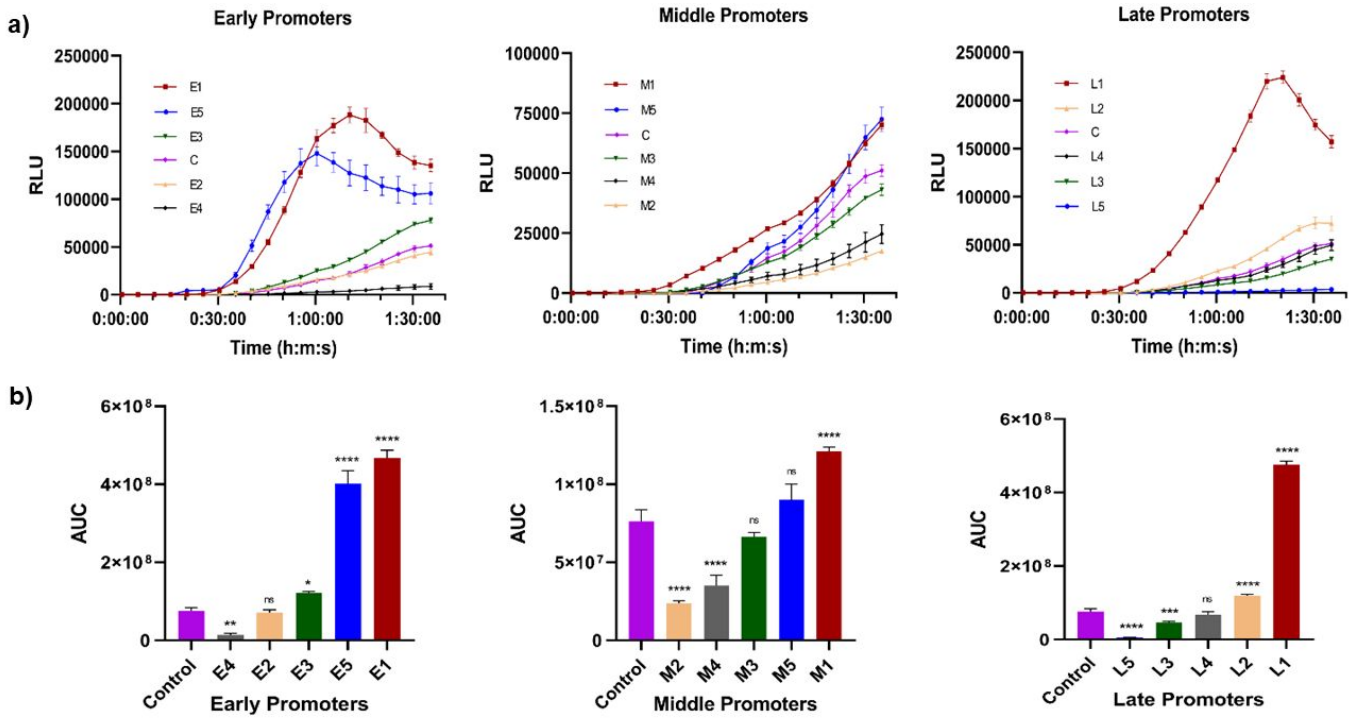

Figure 2: Evaluation of native T4 promoters as indicated by luminescent output from reporter mutant phages. a) Cumulative luminescent output of the 15 tested native promoters categorized into three classes: early, middle, and late. Luminescent output is measured in RLU over 90 mins. b) AUC as a quantitative determination of total RLU as a function of RLU over time (90 mins). Error bars indicate standard deviation of three experimental replicates. Letters and stars indicate significance (ns = $p > 0.05$; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$) by one-way ANOVA and a post-hoc Dunnett's test versus a control were used to determine significance at $p < .05$.

The early promoters are well studied and have been quantitatively evaluated for promoter strength based on *in vitro* expression of β-lactamase and 6-phosphogalactosidase via the pKWIII probe vector model. The pKWIII probe vector promoter strength analysis is based on the enzymatic turnover of ampicillin and ONPG-6-P by β-lactamase and 6-phosphogalactosidase.[40] The five early native T4 promoters with the highest pKWIII value were selected for this study (Table 1). The middle promoters are less widely studied and as a result, five native middle promoters were selected based on canonical inference of translated protein product size. All proteins that are transcribed and translated via the initiation of the middle promoters were compared (Table 1).

Finally, five strong late promoters were selected among the 20 known strong late promoters.[41] The late promoter candidates were selected based on the protein copy number under the assumption that protein level and transcription level behave linearly. Collectively, we used estimates of promoter strengths from several studies which focused separately on early, middle, or late promoters.

Vector Design to Evaluate T4 Promoters

A donor DNA construct was designed to include a unique promoter upstream of the reporter gene, *nluc:cbm*. The reporter is composed of NanoLuc® luciferase (Nluc) fused to a carbohydrate binding module (cbm) for cellulose filter signal capture.[42, 43] In addition, a strong synthetic terminator is added downstream to isolate the output luminescent signal. The reporter sequence was flanked by ~1000 bp regions of homology adjacent to *soc* to provide a template for homologous recombination following CRISPR/Cas9 *soc* cleavage. Golden gate cloning with BsaI restriction sites was added upstream of *nluc:cbm* to allow for seamless incorporation of unique promoter sequences into the base vector construct. Overall, CRISPR/Cas9 mediated phage engineering via the donor vector design creates a homogeneous comparison platform of the upregulation in Nluc production across all 15 inserted promoters.

Evaluation of Promoter Strength Based on Luminescence

Sixteen mutant phages were created in this study, one control reporter T4 phage (NRGp42) without an added promoter and fifteen additional T4 phages (NRGp25-27 and NRGp30-41), each with a uniquely added promoter sequence spanning across three classes of promoter. All mutant phages were further categorized and analyzed within its promoter class. The genetic engineering of all phages was confirmed with whole genome sequencing.

Early – The luminescent outputs of the reporter phages with an added early promoter were compared to the promoter strength reported by Wilkens and Ruger.[40] The Relative Luminescence Unit (RLU) outputs from this study do not correspond with the promoter strength evaluation from previous *in vitro* experiments, an indication that our *in vivo* platform is providing a more accurate depiction of the

systemic effect of promoter-dependent reporter protein upregulation compared to the *in vitro* system. Among the five evaluated early promoters, the promoter sequence that initiates *motB* (E1) transcription generates the highest Nluc signal, resulting in a six-fold increase relative to the control. Upregulation by the added *motB* (E1) promoter generates a luminescent signal with an area under the curve (AUC) of 4.67×10^8 relative to the control, which has an AUC of 7.63×10^7. The promoter sequence for *ndd* (E5) also improves the luminescent output by 5-fold with an AUC of 4.02×10^8 (Figure 2).

Middle – Overall, the middle promoters are the least effective at improving the RLU relative to the early and late promoters with AUC ranging from 2.36×10^7 to 1.21×10^8 (Figure 2). Among the five middle promoters, the promoter sequence of *gp34i* (M1) yields the maximum signal output and improved Nluc signal by 50% as compared to the control. There are several possible reasons why the middle promoters do not perform as well relative to the early or the late promoters: (1) in general, there are fewer middle promoters (30) identified within the T4 phage's genome, as opposed to early promoters (39) , and late promoters (50), (2) middle gene transcription is protein-dependent on two essential protein products (AsiA and MotA) from early gene transcription and (3) as demonstrated in previous work, late promoters outcompete middle promoters,[44] namely that phage encoding proteins gp33, gp45, and the σ factor gp55 direct the RNA polymerase to late promoters and outcompete the AsiA-σ^{70} heterodimers required for middle gene transcription.

Late – The luminescent outputs from the added late promoters unexpectedly do not correspond with the copy number selection criteria. Initially, we hypothesized a linear behavior in the rate of gene transcription and protein translation, hence promoters that correspond to a higher protein copy number would best magnify the Nluc signal. Hypothetically, promoters that initiate *gp23* (L3) and *soc* (L5) would generate the highest Nluc signal. However, these two promoters suppress Nluc production and generated less luminescent signal relative to the control reporter phage. On the other hand, the promoter that initiates transcription of *gp18* (L1) was the best performing among the five promoters but also has the lowest protein copy number. The addition of promoter sequence of *gp18* (L1) resulted in RLU that is six-fold brighter than all the other promoter sequences in its class with an AUC of 4.75×10^8 (Figure 2). We previously expected that the promoter sequences for capsid structural proteins such as *gp23* (L3) and *soc* (L5) would magnify Nluc output based on the high copy number required to assemble the phage particle; however, our overall results indicate otherwise. This phenomenon could be explained by the nonlinear relationship between transcription and translation.

Furthermore, we employed *nluc* as a normalizing factor in our study and thus we were able to attribute the differences in Nluc expression to promoter strength. In addition, we normalized the stability of the *nluc* transcript by using a universal ribosomal binding sequence (RBS). It is possible that the native *gp18* transcript is natively unstable due to a weaker RBS or other stabilization factors, resulting in a lower translation initiation rate and frequency relative to that of *soc* or *gp23*, and further explaining the lower protein copy number.

Conclusions

There is currently a need to improve phage-based biosensors with greater sensitivity to meet regulatory detection limits. We sought to address this need by improving the signal sensitivity of our reporter T4 phages by upregulating transcription of *nluc*. As part of our process, we developed an effective platform to evaluate and compare multiple native promoter activities within our reporter T4 phages. The combination of CRISPR/Cas9 assisted T4 phage engineering coupled with golden gate cloning allows for seamless integration of selected promoters and enables *in vivo* evaluation of promoter activity. As a result, we were able to upregulate Nluc signal by six-fold from the individual addition of a native promoter upstream of the reporter gene. This successful upregulation further optimizes the applicability of reporter phages in phage-based biosensors.

T4 phage gene expression is precisely controlled and is classified into early, middle, and late temporal class. Combining T4 phage promoter activity data from this study with the current classification system will allow us to manipulate the timeline of signal production for phage-based biosensors. We previously engineered a Nluc T4 phage that could detect <10 CFU/100 mL of *E. coli* in drinking water in approximately 7 hours in which *nluc* replaced the nonessential *soc* gene and hence also regulated by the native promoter of *soc*.[34] The results presented in this study suggest that upregulating transcription via an added promoter will improve the assay sensitivity by six-fold, further reducing the current limit of detection and the 7-hour assay time. Collectively, initial *in vivo* promoter analyses indicate that when the reporter gene is placed under a multiplex promoter system, reduced detection times and intensified detection signals throughout the infection cycle are possible.

Overall, our work provides a novel platform that enhances the applicability of phage-based biosensors to mitigate bacterial threats in our food and water systems. As phage-based biosensors are still in early stages of development, increased sensitivity will offer further innovations to this platform for practical application. With respect to T4 phages, transcription and translation regulation is complex and other factors in addition to the ones proposed in this paper should be considered to upregulate protein output. Evaluation of native promoter activity is an initial effort at improving the downstream reporter signal of a phage-based biosensor. Inputting other factors involved in transcription and translation can further improve biosensor sensitivity; for instance, evaluating native and synthetic Ribosomal Binding Sequence (RBS) to directly upregulate protein translation, utilizing the overexpression of enhancers or integrating synthetic or heterologous promoters each is a promising future path to further maximize signal sensitivity.

As a better understanding of phage molecular biology is obtained through evaluation of such factors, new frontiers in phage engineering will become available, such as fabricating customized phages for medical applications such as phage therapy and vaccine development. Collectively, this work begins to tap and actualize the wide application potential and versatility of engineered phages to help address an array of bacterial threats.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

The authors gratefully acknowledge the Bill and Melinda Gates Trust for their sponsorship through the Intellectual Ventures' Global Good Fund. This work is also supported by the AFRI NIFA Fellowships Grant Program: Predoctoral Fellowships, 2019-67011-29611 from the USDA National Institute of Food and Agriculture.

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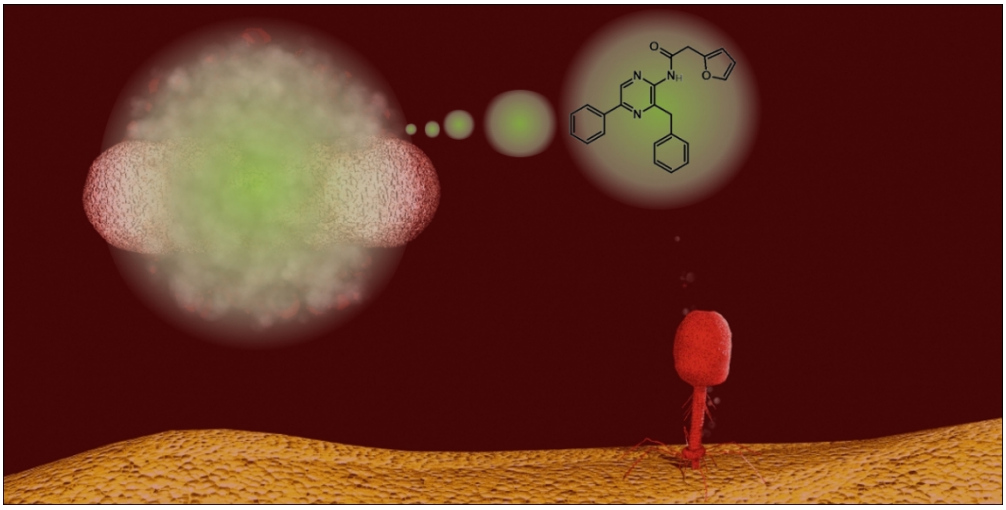

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