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A Thesis for the Degree of Master of Science

Development of an Engineered Bioluminescent Reporter Phage for the Rapid and Sensitive Detection of Viable *Salmonella* Typhimurium

살모넬라 티피뮤리움 생균을 빠르고 민감하게 검출하는 생물발광 리포터 파지의 개발

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Seongmi Kim

Department of Agricultural Biotechnology

Seoul National University

ABSTRACT

Because foodborne illnesses continuously threaten public health, rapid and sensitive detection of pathogens in food has become an important issue. As an alternative to time-consuming and laborious conventional detection methods, a technique using recombinant reporter phages has been developed. Here, we developed an advanced bioluminescent reporter phage SPC32H-CDABE by inserting a bacterial *luxCDABE* operon into the *Salmonella* temperate phage SPC32H genome. Whole SPC32H genome sequencing enabled the selection of nonessential genes, which can be replaced with approximately 6-kb *luxCDABE* operon, which provides both luciferase (LuxAB) and its substrate, fatty aldehyde, as generated by fatty acid reductase (LuxCDE). Thus, the SPC32H-CDABE detection assay is simpler and more efficient compared to the *luxAB*-based assay because the substrate addition step is excluded. At least 20 CFU/mL of pure *S.* Typhimurium culture was detectable using SPC32H-CDABE within 2 h, and the signals increased proportionally to the number of cells contaminated in lettuce, sliced pork, and milk. These results thereby demonstrate that this phage successfully detects live *Salmonella* without appreciable interference from food components. Furthermore, the presented data suggest that

SPC32H-CDABE represents a promising easy-to-use diagnostic tool for the detection of *Salmonella* contamination in food.

Keywords: reporter phage, *luxCDABE*, bioluminescence, *Salmonella* Typhimurium, Foodborne pathogen detection

Student Number: 2012-23370

CONTENTS

I. INTRODUCTION

Salmonella enterica serovar Typhimurium is a Gram-negative pathogen responsible for symptoms that range from mild diarrhea to acute gastroenteritis in humans and severe systemic typhoid fever in animals (1). *Salmonella* species are estimated to cause 93.8 million of gastroenteritis with 155,000 deaths annually worldwide, and 80.3 million cases of these cases were foodborne (2). Because foodborne illnesses caused by *Salmonella* continuously threaten public health, pathogens in food need to be rapidly detected and controlled before consumption. The detection of pathogens in food samples normally relies on culture-dependent methods, which require colony formation of the target bacteria on a growth medium. This can be too time-consuming for food samples, whose quality declines rapidly (3). Polymerase chain reaction (PCR) and enzyme-linked immune sorbent assay (ELISA) have been used as rapid detection methods in place of conventional colony quantification (3). Oligonucleotide probes or antibodies specific for target pathogen DNA or antigens have been used in both methods, respectively. However, a major drawback of these methods is the inability to distinguish live from dead cells. In addition,

ELISA is considered an insufficient tool with respect to sensitivity and specificity because it requires a large number of bacterial cells for reliable signals and has the potential for cross-reactivity with closely related bacteria (4).

One of the alternative pathogen detection methods is the use of a bacteriophage-based reporter system (3). Phage engineering by the insertion of reporter genes, such as *gfp*, *lux*, and *lacZ* genes, into the phage genome enables confirmation of the presence of the target pathogen in the contaminated foods, as assessed using amplified reporter-specific signals (5). The major benefit of using a bacteriophage is the higher specificity for target bacteria and the capacity to differentiate viable cells from nonviable cells (6). Moreover, easy mass production, long shelf-lives, and low cost are comparative advantages of the phage-based reporter system over other detection methods (7). Among the reporter genes used in the phage-based reporter system, *luxAB* genes have several advantageous features over other systems. Luciferase encoded by *luxAB* produces bioluminescence, a physical signal, instead of a chemical signal, thus avoiding the toxicity or instability of the accumulated signal product, which is exhibited by a *gfp*-based system (8). Furthermore, light emission can be precisely and

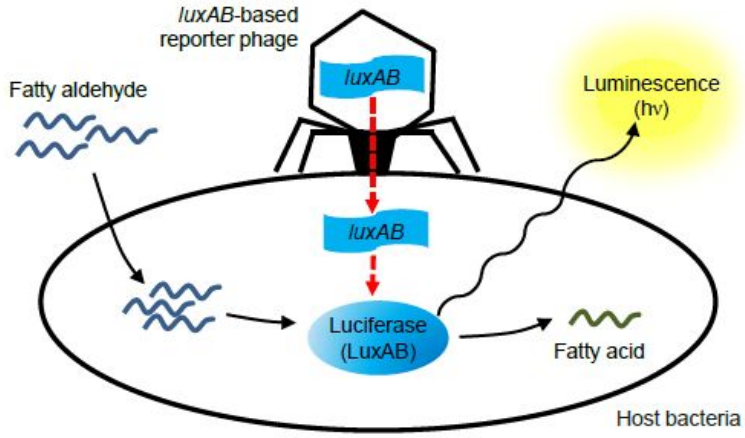
sensitively detected with an extremely low background noise in most sample types (3).

To date, bioluminescence reporter phage systems have been developed using the *luxAB* genes (9-12) without the *luxCDE* genes responsible for the production of luciferase substrate, fatty aldehyde. Thus, exogenous substrates for luciferase have to be supplied with the *luxAB* reporter phage to compensate for the lack of *luxCDE* genes (Figure 1), or both *luxI* reporter phage and *luxCDABE* helper *E. coli* cells were simultaneously applied to detect target bacteria (13). However, the requirement of these secondary components (i.e., substrates or helper cells) other than reporter phage makes the system more laborious and susceptible for user error (12). A one-time measurement immediately after the substrate addition is another limitation of the *luxAB*-based system.

The relatively large size of the *luxCDABE* operon (approximately 6 kb) makes it difficult to clone it into the genome of infectious phages due to the limit of its head capacity (14, 15). Here, we constructed a SPC32H-CDABE reporter phage by replacing the nonessential genes of *Salmonella*-specific temperate phage SPC32H with the bacterial *luxCDABE* operon. This engineered reporter phage

rapidly and sensitively emits bioluminescence when it specifically detects viable *S.* Typhimurium cells from artificially contaminated lettuce, sliced pork, and milk samples, without the addition of any luciferase substrates.

(A)


luxAB-based
reporter phage
luxAB
Fatty aldehyde
Luminescence
(hν)
luxAB
Luciferase
(LuxAB)
Fatty acid
Host bacteria

(B)

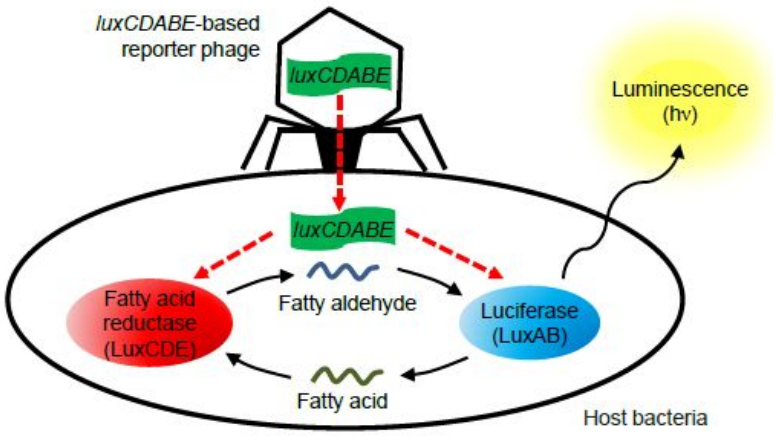

luxCDABE-based
reporter phage
luxCDABE
Luminescence
(hν)
luxCDABE
Fatty acid
reductase
(LuxCDE)
Fatty aldehyde
Luciferase
(LuxAB)
Fatty acid
Host bacteria

Figure 1. Schematic representation of the luminescence reporter phage system. (A) *luxAB*-based reporter phage requires a supplementation of fatty aldehydes, which are the substrates for luciferase (LuxAB) to emit luminescence. (B) *luxCDABE*-based reporter phage supplies fatty aldehydes by themselves using *luxCDE*-encoded fatty acid reductase.

Ⅱ. MATERIALS AND METHODS

Bacterial strains, bacteriophages, and growth conditions

The bacterial strains and bacteriophages used in this study are listed in Table 1. The bacterial strains were routinely grown at 37°C aerobically in Luria−Bertani (LB) broth or LB agar plates [1.5% (w/v) agar], unless mentioned otherwise. When necessary, antibiotics or chemicals were supplemented to the media at the following concentrations: ampicillin (Ap), 50 μg/mL; kanamycin (Km), 50 μg/mL; chloramphenicol (Cm), 25 μg/mL; and L-(+)-arabinose, 100 mM (final concentrations). The O12-2-antigen deficient *S.* Typhimurium LT2(c), which was lysogenized by phage SPC32H [Δ^{LT2}*gtrABC1* (32H), SR5100] (16), was used to engineer the SPC32H genome (*see below*). The engineered phages were induced from the host chromosome using mitomycin C (MMC; 1.0 μg/mL) treatment and then purified, propagated with the appropriate host bacteria, and concentrated using CsCl-density gradient ultracentrifugation as previously described (17). A phage-mediated bacterial challenge assay with *Salmonella* cultures at the early exponential phase was performed

at a multiplicity of infection (MOI) of 5 to confirm the phage infectivity (17).

Table 1. Bacterial strains and bacteriophages used in this study

Bacterial strains	Relevant characteristics[a]	Source or reference
Salmonella enterica serovar Typhimurium		
LT2(c)	Prophage cured *Salmonella enterica* serovar Typhimurium LT2; wild-type	1
MG1655	*Escherichia coli* K-12; wild-type	Laboratory collection
SR5100	Δ^{LT2}*gtrABC1* (SPC32H)	2
SR5513	Δ^{LT2}*gtrABC1* (SPC32H *cps-luxAB*)	This study
SR5167	Δ^{LT2}*gtrABC1* (SPC32H Δ*int*)	This study
SR5519	Δ^{LT2}*gtrABC1* (SPC32H Δ*int cps-luxAB*)	This study
SR5522	Δ^{LT2}*gtrABC1* (SPC32H Δ*og*)	This study
SR5544	Δ^{LT2}*gtrABC1* (SPC32H Δ*int* Δ*og*)	This study
SR5537	Δ^{LT2}*gtrABC1* (SPC32H Δ*int* Δ*og cps-luxAB*)	This study
SR5552	Δ^{LT2}*gtrABC1* (SPC32H Δ*int* Δ*og cps-luxCDABE*)	This study
Escherichia coli		
DH5α lambda *pir*	*supE44* D*lacu169 (*f*80 lacZDM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 λpir*	Laboratory collection
S17-1 lambda *pir*	*recA thi hsdRM*$^{+}$ RP4::2-Tc::Mu::Km Tn7, lambda *pir*; Tp^{R} Sm^{R}	3
Bacteriophages		
SPC32H	Wild type, infect *S.* Typhimurium; O-antigen-	2

	specific	
SPC32H-ABi-1	SPC32H *cps-luxAB*	This study
SPC32H-ABi-2	SPC32H Δ*int cps-luxAB*	This study
SPC32H-AB	SPC32H Δ*int* Δ*og cps-luxAB*	This study
SPC32H-CDABE	SPC32H Δ*int* Δ*og cps-luxCDABE*	This study

a, Km^R, kanamycin resistant; Ap^R, ampicillin resistant; Cm^R, chloramphenicol resistant; Tp^R, trimethoprim resistant; Sm^R, Streptomycin resistant

Engineering of phage SPC32H

For in-frame phage gene(s) deletion, the one-step inactivation method was applied to the pKD46-harboring SPC32H lysogen [Δ^{LT2}*gtrABC1* (32H)] as previously described (16). Briefly, the Km^R-cassette amplified from pKD13 with the appropriate oligonucleotides that annealed to the flanking regions of the deletion-target sites was introduced into the pKD46-harboring SR5100 strain. Positive transformants grown on the Km plate were transformed with pCP20 to excise the Km^R-cassette. The generated strain contained a target gene deletion but also possessed a single FLP recombinase target (FRT) site as a scar. The same method was sequentially repeated to delete other gene(s). The constructs were confirmed by PCR analyses and DNA sequencing of PCR products. To construct a transcriptional *cps-lacZ* fusion, one-step inactivation targeting the site immediately after the *cps* operon was conducted as described above, and then, a promoterless *lacZ* gene from plasmid pCE70 was introduced into the FRT site on scar as described elsewhere (18).

To insert the coding sequence of *luxAB* or *luxCDABE* genes from the reporter plasmid pBBRlux (19) into the SPC32H genome, the specific insertion target site was first inserted by the KmR cassette via one-step

inactivation methods. The sequential overlap PCR amplification of the left/right-flanked fragments (~600 bp) of the target site with a central *luxAB* or *luxCDABE* sequence generated the inset fragment, and this fragment was cloned into the suicide conjugal plasmid pDS132 (20), resulting in donor plasmid pDS-*luxAB* or pDS-*luxCDABE*. *Escherichia coli* S17-1 λ_{pir} strain harboring the donor plasmid and SPC32H lysogen, which contained the Km^R at the target site, was conjugated as described elsewhere (21). The Km^R Cm^R positive conjugant generated by the primary homologous crossover recombination was challenged by 20% sucrose to trigger *sacB* gene-mediated secondary recombination, resulting in the replacement of Km^R to *luxAB* or *luxCDABE*. The insertion of *lux* genes in the Km^S Cm^S clones was validated using DNA sequencing. The plasmids and oligonucleotides used in this study are listed in Table 2 and 3, respectively.

Table 2. Plasmids used in this study

Plasmids	Relevant characteristics[a]	Reference
Lambda red recombination		
pKD46	P_{BAD}-*gam-beta-exo oriR101 repA101*^{ts}; Ap^R	4
pKD13	FRT Km^R FRT PS1 PS4 *oriR6Kγ*; Ap^R	4
pCP20	*cI*857 λP_R*flp oripSC101*^{ts}; Ap^R Cm^R	4
pCE70	FRT *tnpR lacZY*$^+$ *oriR6Kγ*; Km^R	5
In-Out method		
pDS132	*R6K ori mobRP4 cat sacB*; Cm^R	6
pBBRlux	*luxCDABE*; Cm^R	7

a, Km^R, kanamycin resistant; Ap^R, ampicillin resistant; Cm^R, chloramphenicol resistant.

Table 3. Oligonucleotides used in this study

Oligonucleotides	Sequence (5′→ 3’)[a]
Construction of pDS132-32H-*luxAB* and pDS132-32H-*luxCDABE*	
32H-cps-luxAB-U-CF1-XbaI	GAT CAA TCT AGA GCG GTA TCA ACC AAC GTC AT
32H-cps-luxAB-U-CR1	GAT AGA ATA TCT CAA TAG ATT TAC ACG CCC AGT TCT TTA C
32H-cps-luxAB-C-CF1	GTA AAG AAC TGG GCG TGT AAA TCT ATT GAG ATA TTC TAT C
32H-cps-luxAB-C-CR1	TTT TAG CAC TTT ATT GAG CGT TAG GTA TAT TCC ATG TGG T
32H-cps-luxAB-D-CF1	ACC ACA TGG AAT ATA CCT AAC GCT CAA TAA AGT GCT AAA A
32H-cps-luxAB-D-CR1-SacI	ATT AAT GAG CTC TAC TGG TAA GCG TAC TGC CA
32H-cps-luxCDABE-U-CF1-Sal1	AAT TAA GTC GAC GCG GTA TCA ACC AAC GTC AT
32H-cps-luxCDABE-U-CR-E-Xho1	ATC CTC GAG TTA CAC GCC CAG TTC TTT AC
32H-cps-luxCDABE-D-CF-	ATA ACT AGT CGC TCA ATA AAG TGC TAA

E-Spe1	AAG C
32H-cps-luxCDABE-D-CR1-SacI	ATT AAT GAG CTC TAC TGG TAA GCG TAC TGC CA
32H-cps-luxCDABE-C-CF_E_Xho1	GCC CTC GAG CAA AAT GGA TGG CAA ATA TG
32H-cps-luxCDABE-C-CR1_E	GTA GAC TGA GAT AAT CAA AC
32H-cps-luxCDABE-C-CF1_E	GTT TGA TTA TCT CAG TCT AC
32H-cps-luxCDABE-C-CR2_E	ACC CTA AAA TAA ATC TCC CT
32H-cps-luxCDABE-C-CF2_E	AGG GAG ATT TAT TTT AGG GT
32H-cps-luxCDABE-C-CR2_E_Spe1	ATA ACT AGT AGG CAA AGG ATA TCA ACT AT

Construction of SR5167

32H-int-Red-F	ATG AAG CAC GTC ATT CTG GCT GAC AGT ACC CGC GCC ATG ATG TAG GCT GGA GCT GCT TCG
32H-int-Red-R	GTG CTC CCC GTT AAT CCA GGA ATC AAC CAT ATC AGC CCA CAT TCC GGG GAT CCG TCG ACC

Construction of SR5522

32H-gtrA-Red-F1	TTA CCC TAA AGG TGA TAA AAT CTA TAA ATA TAA TAA TTC ATG TAG GCT GGA GCT GCT TCG
32H-oac-Red-R1	CAT AAA TTA TAA ACG GCT GCC TGG GCA GCC GTT TAT TTA TAT TCC GGG GAT CCG TCG ACC

Construction of SR5513

32H-cps-Red-F	AGA GAA GAT TGA CGC CCG GCG TAA AGA ACT GGG CGT GTA ATG TAG GCT GGA GCT GCT TCG
32H-cps-Red-R	ATA GAA AAG CGG GGC CGA ATG GCC CCG GCT TTT AGC ACT TAT TCC GGG GAT CCG TCG ACC

a, Restriction enzyme sites are underlined.

Luminometric assay

Serially diluted bacterial cultures (*S.* Typhimurium LT2 or *E. coli* MG1655) or *Salmonella*-eluted broth from the food samples (*see below*) were infected using the constructed reporter phages (10^8 PFU/mL) and continuously incubated aerobically at 37°C. At the indicated time, 200 μL of culture was transferred to a clean borosilicate glass tube (12 by 75 nm; Kartell spa, Noviglio, Italy) and loaded into a luminometer (model Lumat LB 9507; Berthold, Pforzheim, Germany). For SPC32H-AB, 4 μL of 1% n-decanal (in distilled water; final concentration of 1.2 μmol/mL) was additionally supplemented immediately prior to the measurement (22). The luminescent intensity of each sample was recorded for 5 or 10 s of integration time. The bioluminescence was expressed in relative luminescence units (RLU), and the data was normalized with the background light levels of control (cells or phages alone) within a range of approximately 50 to 150 RLU per 10 s. These results were presented as the average of triplet data ± standard deviations (SD).

Detection of artificially contaminated *Salmonella* in food samples

Bacterial cells were cultured in 3 mL of LB broth at 37°C until an OD_{600} of 0.5 (~ 4×10^7 CFU/mL) was reached to prepare the inoculum for the artificial contamination of food samples: iceberg lettuce, sliced pork, and milk. Serially diluted cultures (10-fold; range from ~10 to ~1×10^6 CFU/mL) in LB broth were spiked into each food sample, and the initial cell concentrations were simultaneously quantified by directly plating the cultures on xylose lysine deoxycholate (XLD) or LB agar plates. The spiking of *Salmonella* cultures into food samples was conducted as follows.

Fresh inner leaves of purchased iceberg lettuce (*Lactuca sativa* L.) heads were sliced (approximately 2 × 5 cm) using a sterile cutter knife (23). The absence of *Salmonella* contamination was confirmed using three samples (10 g each) at the beginning of the experiment by direct quantification on the XLD plate. One-milliliter of prepared *Salmonella* inoculum was spotted onto each 10 g of sliced lettuce leaves. After 2 h of drying at room temperature on a clean bench, each of inoculated leaves was homogenized in 90 mL of LB broth for 2 min using a BagMixer 400 Laboratory Blender (BangMixer, Interscience). The homogenized sample

was serially diluted; 0.1 mL of the diluted samples was plated in duplicate on the XLD plate to measure *Salmonella* load in lettuce, and 5 mL of each was moved into a sterile test tube for further assay. Grocery-store-purchased 90.52% lean sliced pork was weighed in 10 g portions and dipped into the prepared bacterial inoculum for 30 min. Each inoculated sample was dried side by side on a clean bench for 1 h prior to the homogenization and plate counting as described above. Five-milliliters of each was transported into a sterile test tube and subjected to the luminometric assay as described. To contaminate the purchased pasteurized milk, 10-fold serial dilutions from 1×10^7 to 1×10^2 CFU/mL of *Salmonella* culture were prepared and 1 mL of each dilution was added to 10 mL of milk samples in a sterilized 15 mL tube. Inoculated milk samples were statically stored at 4°C for 2 h, and then, 10 mL of each sample was homogenized in 90 mL of LB broth as described above. Each homogenized sample was then subjected to the plate counting and the luminometric assay.

Ⅲ. RESULTS

Deletion of unnecessary genes from SPC32H

Phage SPC32H is a *Salmonella*-specific temperate phage belonging to the *Podoviridae* family. It is categorized in the ε15-like phage group because its 38,689 bp-size genome highly resembles those of ε15 and ε15-like phage phiV10 (16). To insert the bacterial *luxCADBE* operon into this phage genome without interfering with headful packaging, several genes, which are not essential for normal SPC32H phage infection, were selected and removed. Because the lysogenic conversion module consisted of genes encoding an O-antigen acetylase and a putative bactoprenol-linked glucose translocase (*oac* and *gtrA*, respectively; abbreviated *og*) was associated with the superinfection exclusion (6), deletion of the lysogenic conversion module would not be detrimental to phage infectivity. Indeed, the efficiency of plating (EOP) of SPC32H was not affected by the absence of *og* (data not shown). An integrase required for the integration of temperate phage genome into the host chromosome was not needed for the phage infection process. The *int* coding sequence was removed from the SPC32H genome, but the partial 5'-sequence containing arm-type binding sites required for the

excision of phage genome (24) remained. Taken together, deletion of *og* and *int* created space of approximately 3 kb for the *lux* insertion into the SPC32H genome (Figure 2A).

Introduction of the *luxCDABE* coding sequence under the *cps* promoter

Because the phage *cps* gene, which encodes a major capsid protein, is vigorously transcribed by its highly strong promoter P_{cps} and upstream phage promoters (25, 26), the SPC32H *cps* operon was selected as the target site for *lux* operon insertion (Figure 2A). The β-galactosidase assay with the *cps-lacZ* fusion lysogen clearly demonstrated that the putative *cps* operon of SPC32H was strongly transcribed under lytic conditions induced by MMC treatment (data not shown). Integration of the *luxAB* or *luxCDABE* operon into the directed target site was performed using the In−Out process as previously described [(20), *see Materials and Methods*]. Compared to the conventional homologous recombination-mediated double crossover method that is normally used to insert gene fragments containing a selectable maker (27), the In−Out method, which utilizes sequential primary and secondary homologous recombination (Figure 2B), can efficiently select marker-less target recombinant clones. The engineered phages, SPC32H Δ*int* Δ*og*

cps::luxAB and SPC32H Δ*int* Δ*og cps::luxCDABE,* were named as SPC32H-AB and SPC32H-CDABE, respectively.

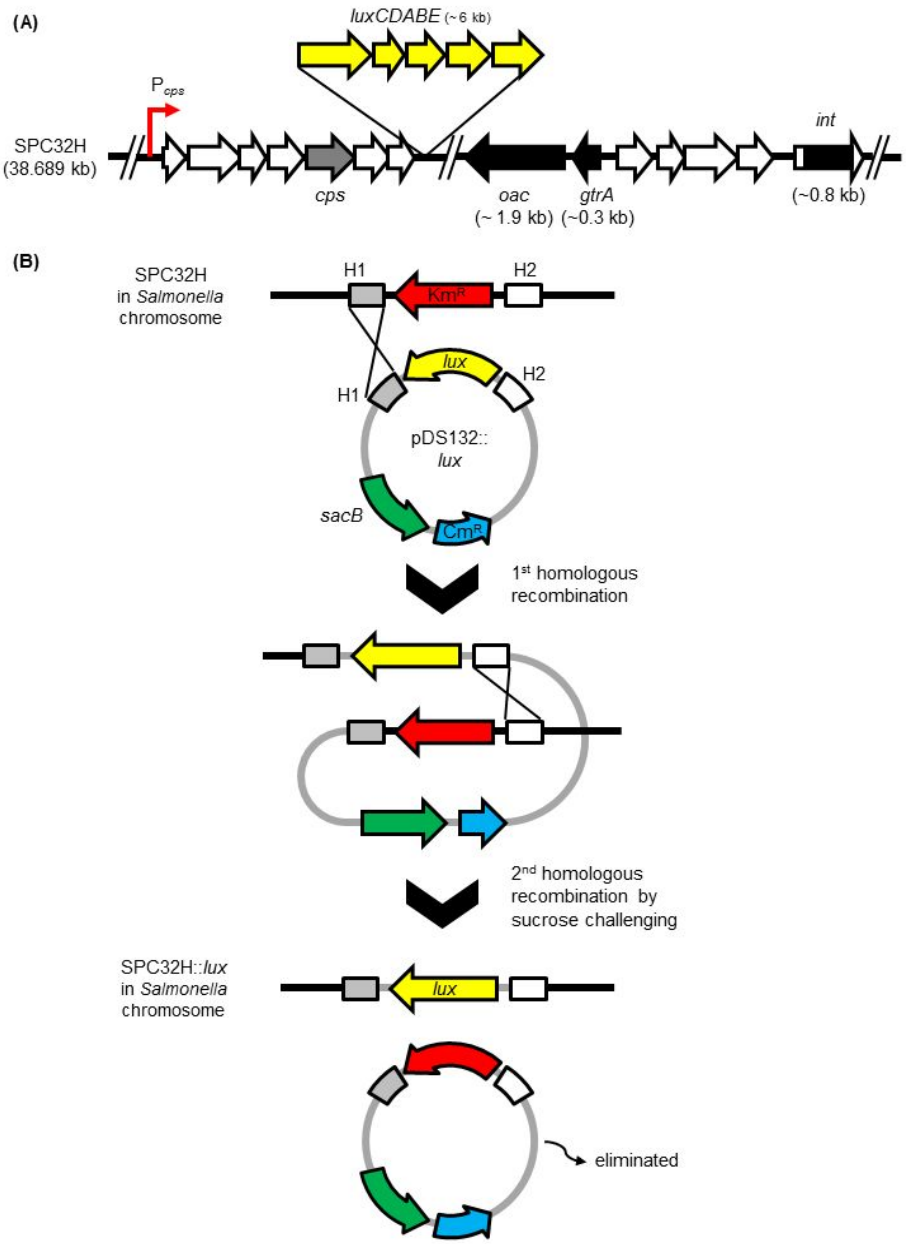

(A)
luxCDABE (~6 kb)
P cps
SPC32H
(38.689 kb)
int
cps
oac
(~1.9 kb)
gtrA
(~0.3 kb)
(~0.8 kb)
(B)
SPC32H
in Salmonella
chromosome
H1
Km^R
H2
lux
H1
H2
pDS132::
lux
sacB
Cm^R
1st homologous
recombination
2nd homologous
recombination by
sucrose challenging
SPC32H::lux
in Salmonella
chromosome
lux
eliminated

Figure 2. Construction of the Bioluminescence Reporter Phage SPC32H-CDABE. (A) Genomic map of engineered SPC32H-CDABE. Whole and partial regions of unnecessary genes (i.e., *oac*, *gtrA*, and *int*; black arrows or box) were deleted prior to insertion of *luxCDABE* operon (yellow arrows) at the downstream of *cps* promoter (red arrow) using the In-Out method (*see panel B*).

(B) Schematic representation of the In−Out method used to insert the *luxAB* or *luxCDABE* operon into the SPC32H genome. pir^{+}-dependent suicide vector pDS132::*lux* was integrated into the chromosome of SPC32H lysogeny using primary homologous recombination, and then, a sucrose challenge facilitated the secondary homologous recombination owing to the *sacB* gene product, resulting in the swapping of the Km^{R} cassette with the *lux* operon.

Fitness of the engineered reporter phage

To examine whether genetic engineering applied to the SPC32H compromised the fitness of the reporter phage, the ability of the engineered phages, including intermediates, to lyse *Salmonella* was analyzed using a bacterial challenge assay. All examined phages caused a significant inhibition of bacterial growth, but no significant bacterial lysis was observed with the *int*-containing phages due to lysogen formation (Figure 3). However, the *int*-deleted phages, SPC32H-AB and SPC32H-CDABE, showed bacterial lysis activity at 1.5 and 2 h after infection, respectively, and the phage resistance development was slower than the *int*-containing phages mainly due to the inability of lysogen formation.

Introduction of *luxAB* into wild type (WT) SPC32H did not significantly affect the phage infection ability (Figure 3), indicating that SPC32H can tolerate an insertion of approximately 2 kb exogenous gene fragment. However, the SPC32H-CADBE (approximately 7.7% more genome than WT) took 30 min longer to start a bacterial lysis than the SPC32H-AB (2.0 vs 1.5 h). Either the complications in phage assembly due to the overexpression of the *luxCDABE* operon from the *cps* promoter or the packaging problem caused by enlarged genome size of SPC32H-CDABE

(approximately 3 kb larger than WT) might delay host lysis. Nevertheless, the level of *Salmonella* growth inhibition by SPC32H-CDABE was comparable to that of SPC32H-AB at approximately 3 h-post infection (Figure 3). Taken together, these results indicated that phage fitness was not compromised by the genetic manipulations previously described, at least in phage infectivity.

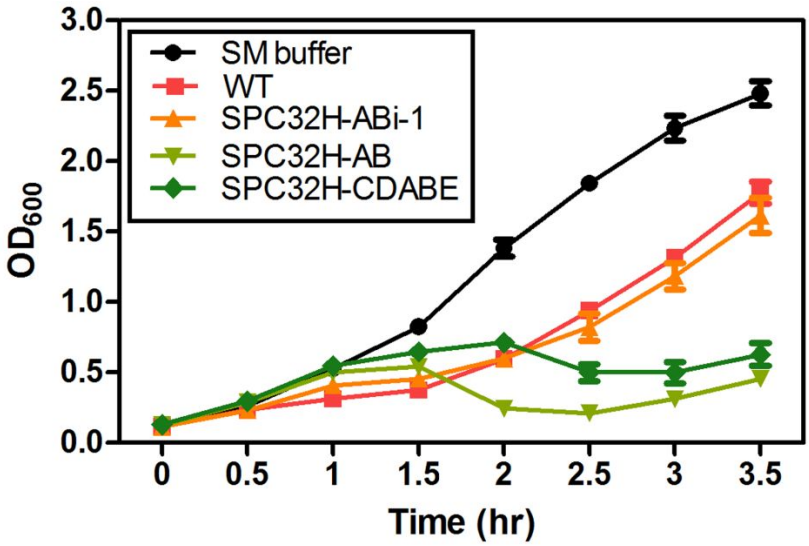


Figure 3. Phage infectivity was not diminished using genomic engineering. *S.* Typhimurium LT2 culture at OD_{600} of 0.11 was infected with various engineered phages and WT phage at an MOI of 5. The OD_{600} was measured at every 0.5 h to monitor the bacterial growth. WT, SPC32H wild type; SPC32H-ABi-1, SPC32H *cps-luxAB*; SPC32H-AB, SPC32H Δ*int* Δ*og cps-luxAB*; SPC32H-CDABE, SPC32H Δ*int* Δ*og cps-luxCDABE*. Another *Salmonella* culture treated with an equal volume of SM buffer instead of phage solution was used as the negative control (SM buffer). The mean of three independent experiments was represented with the standard deviations (SD).

Validation of bioluminescence production using engineered reporter phage infection

When reporter phages, including the intermediates, were used to infect the host *Salmonella*, all examined phages exhibited comparable luminescence (Figure 4), although the bacterial growth inhibitory patterns of these phages were different from each other as discussed above. Importantly, unlike SPC32H-AB, supplementation of n-decanal was not required for phage SPC32H-CDABE to emit bioluminescence after *Salmonella* infection. Generally, bacteriophage has two different life cycles: lytic cycle where an immediate lysis of host cells occurs with viral progeny production and lysogenic cycle where the phage inserts its genome into that of the host and replicates passively at each subsequent host cell division (28). In this context, lysogenic reporter phages, rather than lytic phages, have been known to have a better efficiency in host detection because the lytic phages rapidly destruct the signal production machineries of the host cells (12). Although we deleted the *int* gene from the temperate phage SPC32H (i.e., SPC32H-ABi-2 in Figure 4), the *int* defective phage produced similar levels of bioluminescence as the *int*-containing lysogenic phage (i.e., SPC32H-ABi-1 in Figure 4), indicating that the absence of integrase alone may not dramatically increase lytic activity. Thus, infection of *Salmonella* with the reporter phage SPC32H-

CDABE, which lacks *int,* could demonstrate significant bioluminescence production without immediate lysis of host *Salmonella*. Although integrase is necessary for lysogenic development by temperate phages, deletion of *int* does not drive the lytic cycle because the phage repressor prevents the expression of lytic genes. Thus, the *int* defective phages develop neither a lytic nor lysogenic cycle for a while, such that the Δ*int* phage infected state might resemble the phage carrier state of pseudolysogenic development, which was recently revealed with P22 and *S.* Typhimurium (29).

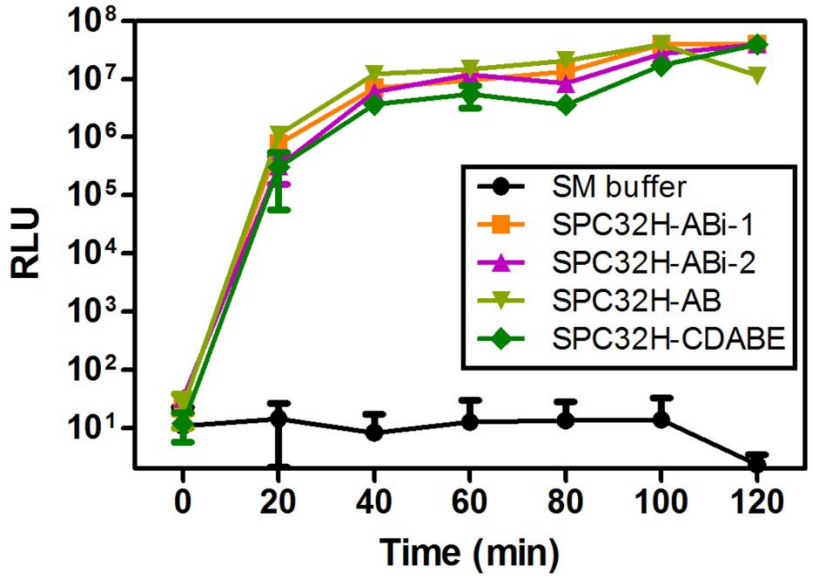


Figure 4. Engineered phage SPC32H-CDABE emits similar levels of bioluminescence to the *luxAB*-based phages without the supplementation of n-decanal. *Salmonella* cultures (~5 × 10^6 CFU/mL) infected with each phage at an MOI of 5 were incubated at 37°C for 2 h. Periodically, the sampled cultures were treated with 1.2 μmol/mL of decanal (final concentration) except the SPC32H-CDABE infected culture, and then, the emitted bioluminescence was measured using a luminometer. SPC32H-ABi-2, SPC32H Δ*int cps-luxAB*. Other figure legends are the same as Figure 3. The results of the mean (n = 3) ± SD were represented.

Reporter phage-mediated specific detection of viable *Salmonella*

To quantitatively measure the efficiency and sensitivity of the reporter phages in *Salmonella* detection, 10-fold serially diluted *Salmonella* cultures were infected by SPC32H-CDABE and the emitted bioluminescence was measured. The phage concentrations (10^8 PFU/mL) and infection time (2 h) used were established to elicit maximum light signals even from small numbers of the host cells (Figures 5 and 6). As low as 20 CFU/mL of *Salmonella* was detectable by infection with the reporter phages in this condition (Figure 7A). Remarkably, a cell number-dependent linear increase of signals was observed with a good correlation between the practical cell numbers enumerated by direct plate counting and RLU levels (R^2>0.98). This result indicated that the SPC32H-CDABE infection accurately reports the number of *Salmonella* cells in the broth by producing a wide linear dynamic range of bioluminescence signals.

As SPC32H was specific to *Salmonella* (16), the engineered reporter phage SPC32H-CDABE also specifically infects several strains of *S.* Typhimurium and *S.* Enteritidis (Table 4). In a practical detection field, such as foods or the environment, there would be numerous types of other bacteria such that the detection method is required not to be disturbed by nonspecific

bacteria. *E. coli* MG1655 cultures, which are selected because of their ubiquitous nature, are mixed with the reporter phages, and no bioluminescence signal was detected (Figure 7A). In addition, the diluted cultures consisting of equal numbers of *S.* Typhimurium and *E. coli* exhibited no interference in the levels of bioluminescence emission by specific *Salmonella* infection (Figure 7A), suggesting that the presence of other bacteria, which are not the host for SPC32H-CDABE, did not interfere with the specific detection of *Salmonella* using the reporter phages.

Since the primary benefit of the phage-based detection system is the discrimination of viable cells from nonviable cells, the ability of SPC32H-CDABE to detect only viable cells was examined. *Salmonella* cultures with 10^5 CFU/mL were divided into two, and only one-half was treated with 70% (v/v) ethanol for 30 min, harvested, and resuspended in the same volume of LB broth (30). Resuspended dead cells and live cells were mixed at different ratios (100%, 10%, 1%, 0.1%, and 0% live cells (v/v)), and each mixture was infected with SPC32H-CDABE (10^8 PFU/mL). Ethanol treatment caused more than a five-log reduction in viable cell numbers (no colonies on plates) and resulted in no bioluminescence from SPC32H-CDABE infection, and the increase of bioluminescence was directly proportional to the amount of live

cells (Figure 7B). These results demonstrate that SPC32H-CDABE could avoid reporting a false-positive result in response to the dead *Salmonella* cells irrespective of the live to dead cell ratios.

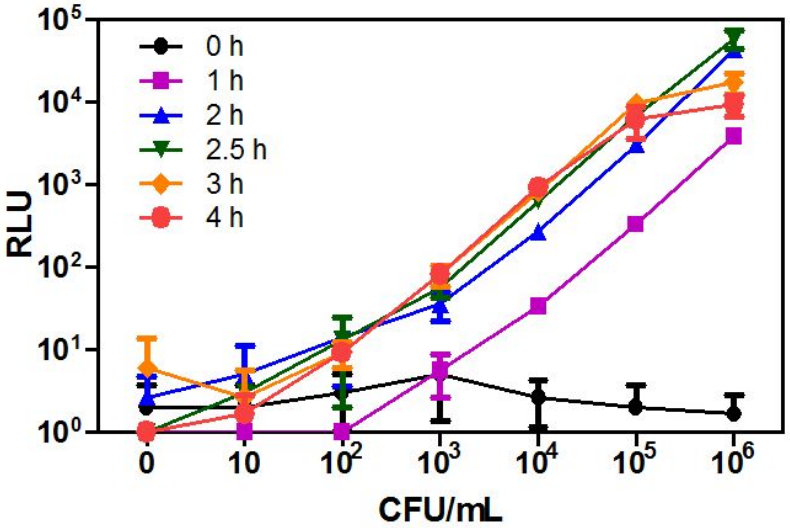


Figure 5. Optimization of infection time is required to verify the number of *Salmonella* in pure cultures. Reporter phages at high concentration (10^9 PFU/mL) were infected into serially diluted cultures of *S.* Typhimurium LT2 and incubated at 37°C for 4 h with periodical sampling for luminometric assay using Tecan (Infinite 200 PRO, Switzerland). Two-hours of infection time was selected for further experiments because it was the minimum amount of time required to exhibit sufficiently high signals that were proportional to the cell number ($R^2 = 0.992$). The results are expressed as the mean (n = 3) ± SD.

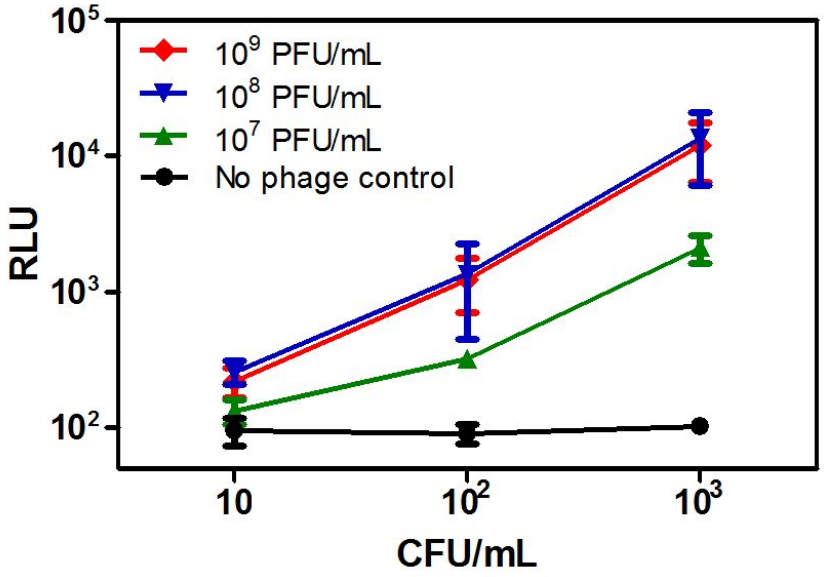


Figure 6. Optimization of phage concentration to detect a low number of *Salmonella* cells. Ten-fold diluted reporter phages were infected into serially diluted cultures of *S.* Typhimurium LT2 and incubated at 37°C for 2 h. The emitted bioluminescence was measured using Tecan. The minimum concentration of 10^8 PFU/mL exhibiting sufficiently high signals was proportional to the cell number was selected for a further infection test. The results are expressed as the mean (n = 3) ± SD.

Table 4. Host range of SPC32H, SPC32H-AB and SPC32H-CDABE

	Phage plaques[a]		
Host	SPC32H	SPC32H-AB	SPC32H-CDABE
Salmonella Typhimurium			
LT2	C	C	C
UK1	C	C	C
SL1344	C	C	C
14028s	C	C	C
ATCC 19586	C	C	C
ATCC 43174	C	C	C
DT104	C	C	C
S. Enteritidis			
ATCC 13078	C	C	C
S. Typhi			
Ty2-b	-	-	-
S. Paratyphi			
A 1B 211	-	-	-
B 1B 231	T	T	T
C 1B 216	T	T	T

S. Dublin			
1B 2973	-	-	-
S. enterica subsp. *arizonae*			
KCCM 41035	-	-	-
KCCM 41575	-	-	-
KCCM 41651	-	-	-
S. enterica subsp. *indica*			
KCCM 41759	-	-	-
S. enterica subsp. *houtenae*			
KCCM 41760	-	-	-
S. enterica subsp. *diarizonae*			
KCCM 41761	-	-	-
S. enterica subsp. *salamae*			
KCCM 41762	-	-	-
S. enterica isolates			
3068	T	T	T
3605	-	-	-
3792	-	-	-
4509	T	T	T

Escherichia coli			
MG1655	-	-	-
MC4100	-	-	-
DH5α	-	-	-
DH10B	-	-	-
O157:H7 ATCC 35150	-	-	-
O157:H7 ATCC 43888	-	-	-
O157:H7 ATCC 43890	-	-	-
O157:H7 ATCC 43894	-	-	-
O157:H7 ATCC 43895	-	-	-
O157:NM 3204-92	-	-	-
O157:NM H-0482	-	-	-

a, C, clear plaques; T, turbid plaques; -, no plaques.

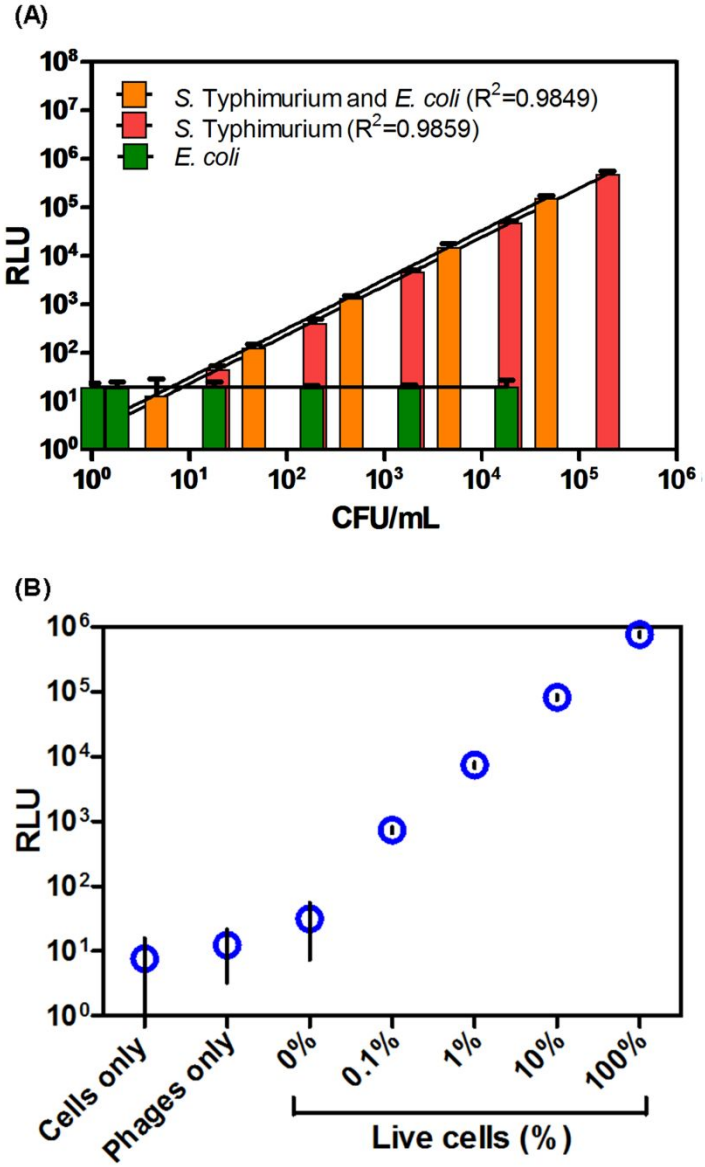

(A)
S. Typhimurium and E. coli (R2=0.9849)
S. Typhimurium (R2=0.9859)
E. coli
RLU
CFU/mL
(B)
RLU
Cells only
Phages only
0%
0.1%
1%
10%
100%
Live cells (%)

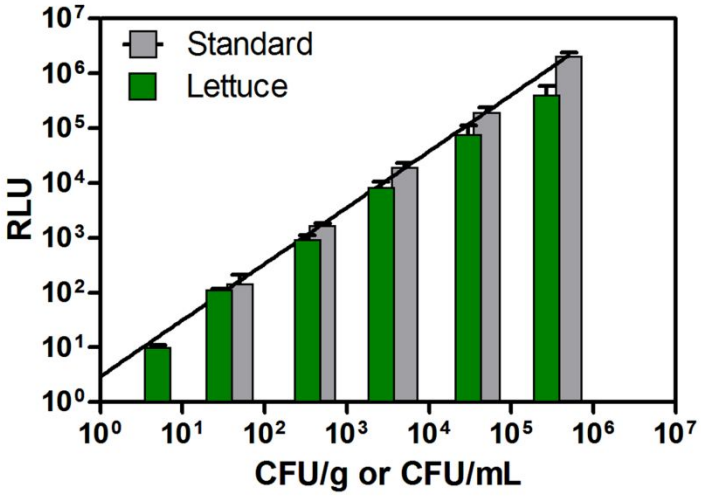
Figure 7. Reporter phage SPC32H-CDABE specifically detects viable *Salmonella* cells. (A) Infection of SPC32H-CDABE emits a bioluminescence proportional to the number of *Salmonella* cells independent of the presence of nontarget bacteria. Phage dilutions (10^8 PFU/mL) were infected with serially diluted cultures of *S.* Typhimurium LT2, *E. coli* MG155, or a mixture of them and incubated at 37°C for 2 h for the luminometric assay. The results are expressed as the mean (n=3) ± SD. (B) Only viable *Salmonella* cells were detected using SPC32H-CDABE. *Salmonella* cultures with 10^5 CFU/mL were divided into two, and only one-half was treated with 70% ethanol for 30 min. The washed dead and live cells were mixed at the indicated ratios. Each sample was infected with SPC32H-CDABE (10^8 PFU/mL), and then the bioluminescence was measured after the 2 h incubation at 37°C. Experiments without cells or phages were also performed as controls. The results of three independent experiments are represented as the mean ± SD.

Food application; lettuce, sliced pork, and milk

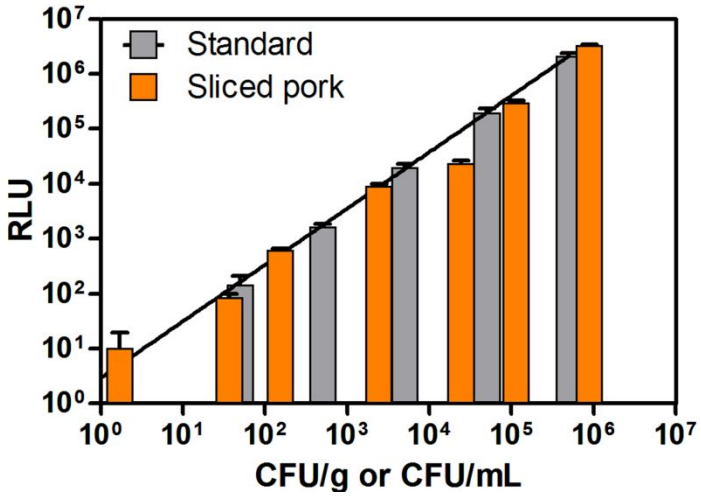
The *Salmonella* detection ability of SPC32H-CDABE was tested using three types of foods, including iceberg lettuce, sliced pork, and milk, which were susceptible to *Salmonella* contamination. Lettuce represents minimally processed ready-to-eat fresh vegetables, which are eaten raw without any heat treatment or reduction method (31). Sliced pork was tested because meat consisting of pork, chicken, and beef is known to demonstrate a strong inhibitory effect on the PCR detection of foodborne pathogens (32). When the reporter phages were mixed with bacteria in artificially contaminated lettuce or sliced pork, significant bioluminescence was produced in a bacterial cell number-dependent manner, and the levels of these signals were comparable to those in the standard curve with the *Salmonella* pure cultures (Figure 8A and B). Because one drawback of *lux*-based reporter phage was an inhibition of luminescence signaling in the turbid sample matrix (3), we examined our reporter phages in turbid food, milk. Consistent with previous reports (3), comparatively lower levels of bioluminescence were emitted from the *Salmonella* contaminated milk compared to other two food samples (Figure 8C). Although the intensity of the light signal was decreased by one-log, the graph still shows linear and dose-dependent characteristics,

suggesting that SPC32H-CDABE can quantitatively report the presence of *Salmonella* in turbid matrix foods. These results demonstrated that SPC32H-CDABE could precisely and rapidly report the number of viable target *Salmonella* cells contaminated in the food samples even though the detection limits were changed differentially depending on the food. The detection limits were 22 (± 10.4) CFU/g for lettuce, 37 (± 10.4) CFU/g for sliced pork, and 700 (± 11.6) CFU/mL for milk (Figure 8). These data were statistically significant when the student's *t* test was used (p <0.05).

(A)


Standard
Lettuce
RLU
CFU/g or CFU/mL

(B)


Standard
Sliced pork
RLU
CFU/g or CFU/mL

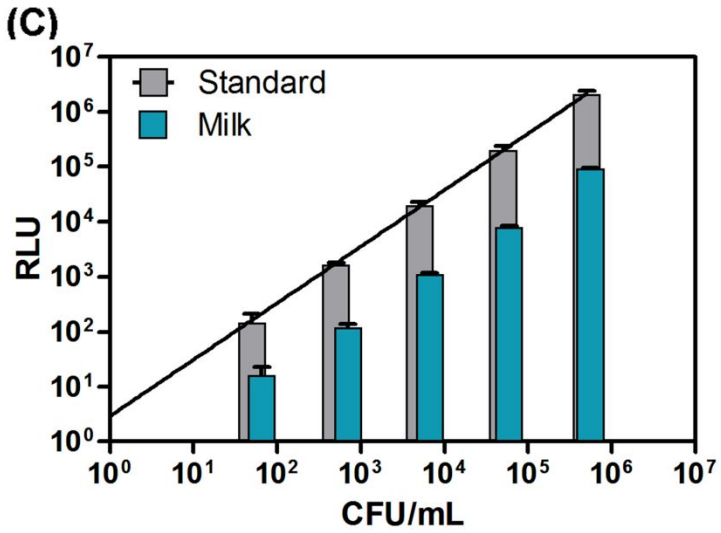


Figure 8. SPC32H-CDABE detects *Salmonella* from artificially contaminated foods. Serially diluted *S.* Typhimurium LT2 cultures were spiked on (A) lettuce, (B) sliced pork, and (C) milk, and each sample was subsequently subjected to *Salmonella* detection using SPC32H-CDABE (*see EXPERIMENTAL PROCEDURE SECTION for more details*). The gray bars indicate the standard curve obtained with pure cultures of *S.* Typhimurium. The results are expressed as the mean ± SD of three independent experiments. Detection limits in this experiment were 22 ± 10.4 CFU/g (lettuce), 37 ± 10.4 CFU/g (sliced pork), and 700 ± 11.6 CFU/mL (milk).

Ⅳ. DISCUSSION

For accurate foodborne pathogen detection, many efforts to develop an effective detection system have continued using diverse approaches. Bacteriophages itself (e.g., phage typing and phage amplification) and engineered forms (e.g., fluorescently labeled phage, reporter gene integrated phage, and phage display) have been used for pathogen detection (12) because they show host-specificity and can distinguish living cells from dead cells (6). In this study, we constructed a *luxCDABE* reporter phage from temperate phage SPC32H, which can be used to easily verify *Salmonella* contamination in foods with high sensitivity. Reporter phage with the whole *luxCDABE* operon enables the elimination of the substrate addition step, thereby enabling the direct detection of light signals without supplementary manipulation (Figure 1).

Despite the advantages of using the whole *luxCDABE* operon, only the *luxAB* reporter phage and *luxI* reporter phage system with *luxCDABE* helper *E. coli* cells (10, 13) have been developed thus far, most likely due to difficulties in incorporating the large *luxCDABE* operon into the phage.

Phages have a specific DNA packaging mechanism, which recognize a *cos* or *pac* site on their genome using the terminase protein (15, 33), and some phages, such as P22, possess a headful packaging system, which is able to tolerate a limited genome size of approximately 102 to 110% (34). Phylogenetic tree analysis of the terminase large subunit (TerL) suggested that SPC32H belongs to the "P22-like headful packaging" group (Figure 9). Insertion of the whole *luxCDABE* operon into the intact SPC32H genome was estimated to increase the genome size by approximately 15%, and consequently, results in a significant reduction in infectivity (10^7-fold decrease in EOP). However, deletion of non-essential lysogen-related genes, such as *int*, *oac*, and *gtrA* from the SPC32H genome created space for the insertion of the whole *luxCDABE* operon without damaging its infectivity (Figure 3). Although the lack of integrase reduced the induction rate of engineered recombinant prophage from the host chromosome, the infectivity and signal production of int^- phages was the same or better than those of int^+ phages (Figure 3 and 4). In addition, insertion of the *luxCDABE* operon under the strong *cps* promoter of the phage resulted in high levels of *lux* transcription for the sensitive detection of bioluminescence (25).

Some pathogen detection methods, such as PCR and various

immunological tests, are not suitable for use in food samples because the complex food matrix containing ATP, nontarget DNA, proteins, and particulates are a critical barrier for the detection of target cells (35, 36). Compared with these methods, the luminescence reporter system detects the target pathogen in foods with extremely low background noise and exhibits 10^2 to 10^3 times more sensitivity (37). The lack of endogenous luciferase activity in organisms and cells except some luminous marine bacteria clearly indicates the widespread use of the *lux* gene as a reporter to detect pathogens in foods. The use of *lux* reporter phages is also advantageous compared to other reporter-based systems, such as the ATP bioluminescence detection system (35), because no reaction condition adjustments for each food are required. Furthermore, the absence of toxicity facilitates the precise measurement of luminescence without a disruption of the cell membrane or loss of cell viability, which prevents false-negative results (8), such that the reporter phage specifically responds to the viable target cells (Figure 7).

The *lux* reporter phage SPC32H-CDABE successfully detected *Salmonella* from artificially contaminated lettuce, sliced pork, and milk (Figure 8), indicating that the sensitivity of *Salmonella* detection in foods with SPC32H-CDABE was not hindered by food components. Although the

infectious dose for *Salmonella* is highly dependent upon the strain encountered and the health condition of the host, the infectious dose at which 50% develop illness (ID_{50}) for *Salmonella* is reported to be 10^7 cells in healthy volunteers (38). Because the SCP32H-CDABE reporter phage detected nearly 2×10 to 7×10^2 CFU/mL (or CFU/g) in food samples, the present methods with SPC32H-CDABE will be useful to monitor the *Salmonella* contamination of food.

The strategy used in this study to construct the *luxCDABE*-based reporter phage (i.e., deletion of unnecessary phage genes using the lambda red recombination, and targeted insertion of *luxCDABE* operon using the In-Out double homologous recombination) was not restricted to the *Salmonella*-specific phage SPC32H, but could be applied to other temperate phages, which are specific for various pathogens. Engineering of the receptor binding protein of phages or the evolutionary host retargeting could also be applied to expand the host range of recombinant reporter phages (39, 40).

The recombinant SPC32H-CDABE reporter phage, which is infectious, similar to the wild type phage, rapidly and sensitively detects viable *Salmonella* cells in both pure cultures and in artificially contaminated foods without substrate supplementation. Considering these advantages, SPC32H-

CDABE and other *luxCDABE*-based reporter phages could be used to facilitate the development of an easy-to-use phage-based pathogen detection system, such as a portable laboratory diagnostic tool kit (7).

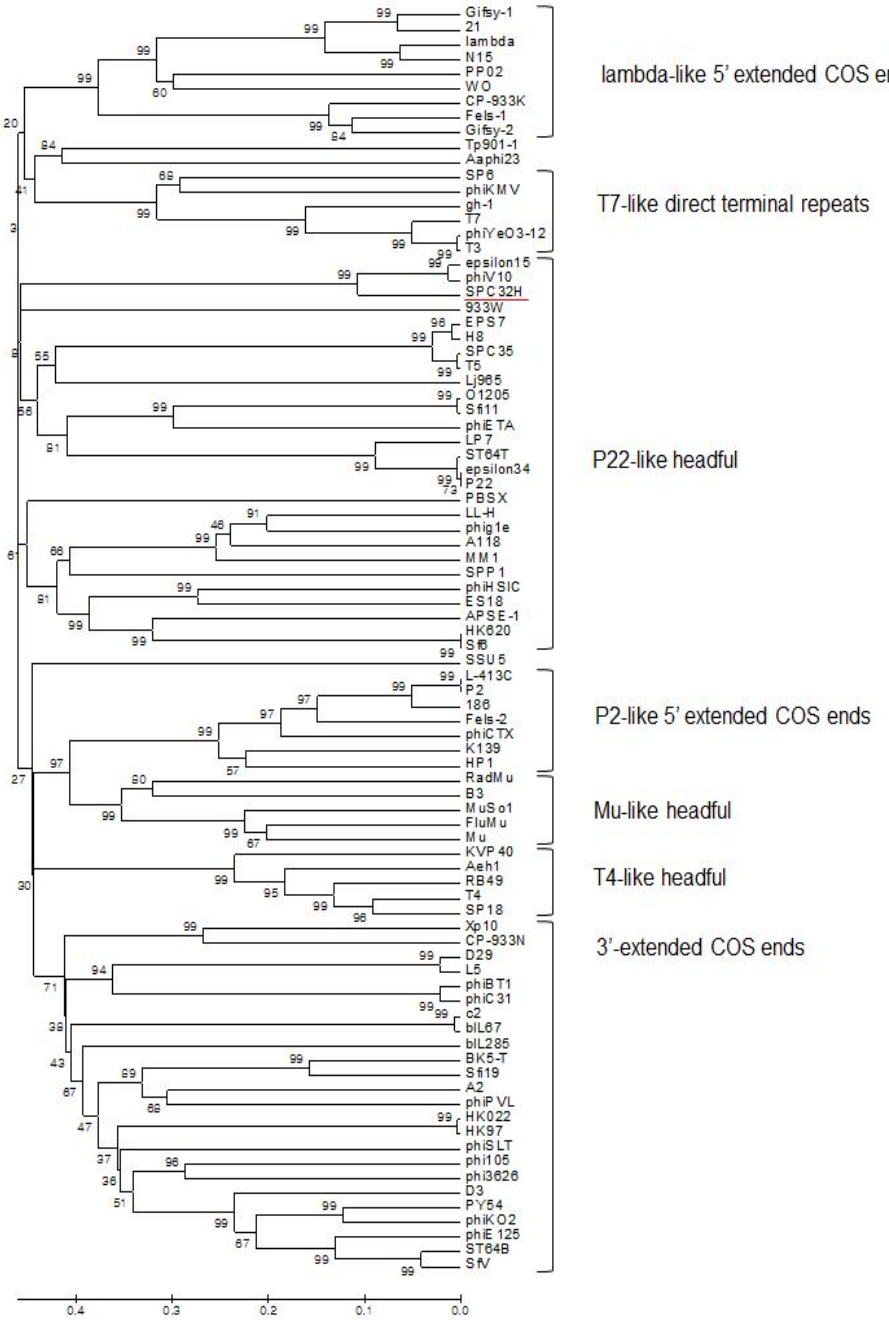

Gifsy-1
21
lambda
N15
PP02
WO
CP-933K
Fels-1
Gifsy-2
Tp901-1
Aaphi23
SP6
phiKMV
gh-1
T7
phiYeO3-12
T3
epsilon15
phiV10
SPC32H
933W
EPS7
H8
SPC35
T5
Lj965
O1205
Sfi11
phiETA
LP7
ST64T
epsilon34
P22
PBSX
LL-H
phig1e
A118
MM1
SPP1
phiHSIC
ES18
APSE-1
HK620
Sf6
SSU5
L-413C
P2
186
Fels-2
phiCTX
K139
HP1
RadMu
B3
MuSo1
FluMu
Mu
KVP40
Aeh1
RB49
T4
SP18
Xp10
CP-933N
D29
L5
phiBT1
phiC31
c2
bIL67
bIL285
BK5-T
Sfi19
A2
phiPVL
HK022
HK97
phiSLT
phi105
phi3626
D3
PY54
phiKO2
phiE125
ST64B
SfV
0.4
0.3
0.2
0.1
0.0
lambda-like 5' extended COS ends
T7-like direct terminal repeats
P22-like headful
P2-like 5' extended COS ends
Mu-like headful
T4-like headful
3'-extended COS ends

Figure 9. Neighbor-joining phylogenetic tree analysis based on the alignment of the amino acid sequences of the terminase large subunit from various bacteriophages. The numbers at the nodes indicate the bootstrap probabilities.

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국문 초록

식중독의 발생이 지속적으로 공중보건을 위협하는 만큼, 식품 내에서 빠르고 민감하게 식중독균을 검출하는 것이 중요한 문제로 대두되고 있다. 재조합 리포터 파지는 시간과 인력이 많이 소모되던 종전의 검출 방법을 앞서는 장점덕분에 새로운 검출 방법으로 긍정적인 평가를 받고 있다. 이에 본 연구에서는 리포터 유전자인 *luxCDABE* 오페론을 살모넬라 temperate 파지 SPC32H 유전체에 삽입하여 좀 더 발전된 재조합 생물발광 리포터 파지를 구축하였다. SPC32H의 유전체 염기서열 분석이 완료되었기에 선택적으로 불필요한 유전자를 삭제하고, 그 공간에 6천 염기 크기의 *luxCDABE* 오페론을 삽입할 수 있었다. *luxCDABE*는 발광단백질인 루시퍼레이즈(luciferase, LuxAB)와 그의 기질인 지방산 알데하이드를 생성하는 지방산 환원효소(fatty acid reductase, LuxCDE)를 인코딩하고 있다. 따라서 *luxCDABE*는 발광단백질의 기질을 첨가할 필요가 없으므로 *luxAB* 리포터 파지에 비해 검출 시험이 훨씬 간단하고 효율적일 수 있다. 본 연구에서는 SPC32H-CDABE 리포터 파지를 이용해 살모넬라균 배양액 1ml 에서 최소 20마리의 살모넬라균을 2시간 내에 검출할 수 있었다. 또한 식품 시료인 양상추, 가공 햄 그리고 우유에서

식품의 복잡한 구성 성분에 의해 저해를 받지 않고, 살모넬라균 수에 비례하여 생물발광이 증가하는 것을 관찰하였다. 따라서 본 연구의 생물발광 리포터 파지를 실제로 식품에 사용하여 오염된 살모넬라균의 수를 보다 정확하고 빠르게 측정할 수 있음을 입증하였다. 또한 이 리포터 파지는 추가적인 기질의 첨가가 필요하지 않으므로 검출 시스템의 편의성도 높였다는 점에서 실제로 식품의 살모넬라균 오염을 검출하는 데 적용될 수 있을 것으로 보인다.

주요어: 리포터 파지, *luxCDABE*, 생물발광, 살모넬라 티피뮤리움, 식중독균 검출

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