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Effective inhibition of *Salmonella* Typhimurium in fresh produce by a phage cocktail targeting multiple host receptors

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ABSTRACT

Salmonella contamination of fresh produce is the primary bacterial cause of a significant number of foodborne outbreaks and infections. Bacteriophages can be used as natural antibacterial agents to control foodborne pathogens. However, the rapid development of bacterial resistance to phage infection is a significant barrier to practical phage application. To overcome this problem, we developed a novel phage cocktail consisting of the three phages (BSPM4, BSP101 and BSP22A) that target different host receptors, including flagella, O-antigen and BtuB, respectively. Whole genome sequence analysis of the phages revealed that three phages do not harbor genes involved in lysogen formation or toxin production, suggesting they are safe for use as biocontrol agents in foods. *In vitro* treatment of the phage cocktail resulted in a significant reduction in the development of bacterial resistance. Phage cocktail treatments achieved 4.7–5.5 log CFU/cm^2 reduction of viable cell number in iceberg lettuce and 4.8–5.8 log CFU/cm^2 reduction in cucumber after 12 h at room temperature (25 °C). The phage cocktail exhibited good antimicrobial efficiency, suggesting that it could reduce *S.* Typhimurium contamination of fresh produce. The strategy of developing cocktails of phages that target multiple host receptors can be used to develop novel biocontrol agents of *S.* Typhimurium.

1. Introduction

Salmonella is a leading bacterial cause of foodborne infection. *Salmonella* is estimated to be responsible for more than 90 million cases of gastroenteritis every year and 155,000 deaths worldwide (Majowicz et al., 2010). Most *Salmonella* infections are self-limiting in healthy people, but can cause serious illness or even death in young and elderly individuals and immunocompromised patients (LaRock et al., 2015). Fresh produce is the major food type involved in foodborne bacterial outbreaks. The Centers for Disease Control and Prevention (CDC) reported that 46% of foodborne illnesses in the US are caused by fresh produce (Painter et al., 2013). Among diverse food-borne pathogens, *Salmonella* is one of the most worrying pathogen associated with fresh produce because of its strong persistence and survival in the environment (Dhokane et al., 2006; Scallan et al., 2011). For example, a recent multistate *Salmonella* outbreak due to contaminated cucumbers in the US in 2015 resulted in 204 hospitalizations and six deaths (CDC, 2016). Therefore, it is vital to control *Salmonella* in fresh produce for food safety and public health. Unlike meat products that are cooked at high temperatures before consumption, thermal treatment options for fresh produce are limited; thus, chemical antimicrobial compounds [e.g., chlorine, chlorine dioxide (ClO_2), ozone (O_3), and peracetic acid (PAA)] are widely used in the food industry to reduce the chances of cross-contamination in fresh produce (Fernandez et al., 2013; Kim et al., 2008). However, the generation of hazardous by-products, such as trihalomethanes (THMs), haloacetic acids (HAAs), chlorite, chlorate and bromate, are of concern to consumers, and the residues of those chemical compounds need to be monitored during application (Banach et al., 2015). Given increasing food safety concerns about fresh produce and limited treatment options to disinfect fresh produce, novel and safe methods of improving the safety of fresh produce are urgently required (Jones et al., 2010).

Bacteriophages (phages) are self-replicating virus particles with bacterial lysis activity, and can infect and lyse specific host bacteria without additional treatment (Chang et al., 2015; Kutter and Sulakvelidze, 2005). Phages infect only bacteria and are known to not be harmful to humans, making them safe for treatment of food products (McCallin et al., 2013). Moreover, phages do not affect properties of

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food products such as flavor, color, and taste (Pietracha and Misiewicz, 2016), making phages an attractive alternative biocontrol agent in foods.

Despite the aforementioned advantages of using phages as biocontrol agents, the emergence of phage-insensitive bacteria strains has limited their efficacy (Labrie et al., 2010).

To overcome this problem, Tanji et al. suggested a phage cocktail composed of two phages targeting different receptors including an outer membrane protein OmpC and lipopolysaccharides (LPS), and it successfully reduced the emergence of phage-resistant *Escherichia coli* O157:H7 (Tanji et al., 2004). They also developed a cocktail containing three phages without knowing the receptor of one phage that showed a significant reduction in *E. coli* O157:H7 concentration in gastrointestinal tract of mice (Tanji et al., 2005). In addition, treatment of a phage cocktail containing 37 phages efficiently reduced *E. coli* O157:H7 in calves without the development of phage resistant mutants (Villa and Crespo, 2010). However, preparation of a cocktail comprised of 37 different phages is not practical and new strategies for phage cocktail preparation are required to design efficient phage cocktail.

Salmonella often develops resistance to phage infection, usually by modifying surface receptors (Labrie et al., 2010). It has been reported that the O-antigen-targeting phages could not infect the *S*. Typhimurium resistant to the BtuB-targeting phage and the BtuB-targeting phages could not infect the *S*. Typhimurium resistant to the O-antigen-targeting phages (Kim and Ryu, 2012; Shin et al., 2012). These results suggest that cross-resistance development among phages using different receptors should also be considered and receptor identification of the phages used for making phage cocktail is essential to avoid this problem.

In this study, we identified receptors of the isolated phages and made a phage cocktail comprised of three phages, which use different receptors. *Salmonella* did not develop cross-resistance against these three phages and emergence of *Salmonella* resistant to the phage cocktail was delayed. The phage cocktail showed efficient control of *S*. Typhimurium contamination on fresh produce.

2. Materials and methods

2.1. Bacterial strains, media, and growth conditions

Bacterial strains used in this study are listed in Supplementary Table S1. All of these strains were grown aerobically in Luria-Bertani (LB) broth medium (Difco, Detroit, MI, USA) at 37 °C or at 30 °C in some cases. LB agar plates were prepared with LB broth supplemented with 1.5% Bacto agar (Difco), and soft top agar containing LB broth was prepared with 0.4% agar for phage plaque confirmation. For phage isolation, prophage-cured *S.* Typhimurium LT2 strain (designated LT2C) and its derivative mutants were used as hosts to exclude prophage induction.

2.2. Bacteriophage isolation, purification, and propagation

S. Typhimurium-targeting bacteriophages were isolated from environmental samples (Table S2). Bacteriophages were isolated as described previously with slight modifications (Park et al., 2012). Briefly, 25 g of each sample was mixed with 225 mL sterile Butterfield's phosphate-buffered dilution water (0.25 M KH_2PO_4 adjusted to pH 7.2 with NaOH) in sterile bags and then homogenized for 90 s using a BagMixer 400 blender (Interscience Laboratory Inc., St. Nom, France). Twenty-five milliliters of each homogenized sample was diluted with the same volume of 2X fresh LB broth and incubated at 37 °C overnight with shaking (220 rpm). After incubation, samples were centrifuged at 10,000 × g for 10 min and filtered using 0.22 μm pore size filters (Millipore, Billerica, MA, USA) to remove residual bacteria. Ten milliliters of each supernatant was added to 40 mL of LB broth containing the overnight culture of the host strain (1% final concentration) and then incubated. Cultures were centrifuged at 10,000 × g for 10 min and the supernatant was filtered. To confirm the presence of bacteriophages, supernatants were serially diluted 10-fold and spotted on molten 0.4% LB soft agar containing *Salmonella* (3×10^9 CFU/mL). After drying for 15 min, agar plates were incubated at 37 °C overnight and plaque formation was observed.

For phage propagation, the lysate of a single phage was added at a multiplicity of infection (MOI) of 1 to the culture of the desired host *Salmonella* strain (2 h, OD_{600} = 0.5) and the mixture was incubated at 37 °C for 3 h. Phage lysates were obtained by centrifugation and filtration as described above. Phage propagation steps were serially performed with three different culture volumes (3, 50, and 200 mL culture) to obtain a sufficient volume of phage lysate. To prepare high-titer phage stock, phage particles were precipitated using polyethylene glycol (PEG) 6000 (Junsei) in 1 M sodium chloride (final concentration) at 4 °C for 12 h. After centrifugation (10,000 × g, 20 min, 4 °C), precipitated phage particles were suspended in 1 mL of SM buffer and separated by CsCl density gradient ultracentrifugation for 2 h at 4 °C (himac CP 100β, Hitachi, Japan) using step densities of 1.3, 1.45, 1.5 and 1.7 g/mL and a centrifugation speed of 78,500 × g. Finally, the phage band fraction was collected and dialyzed twice for 1 h in 1 L of standard dialysis buffer (5 M NaCl, 1 M $MgCl_2$ and 1 M Tris·HCl at pH 8.0) using a Spectra/Por dialysis membrane (molecular weight cut-off, 12,000 to 14,000; Spectrum Laboratories, Inc., Rancho Dominguez, CA). Concentrated phage stocks (approximately 1×10^{10} to 1×10^{11} PFU/mL) were stored in a glass vial at 4 °C after phage titer measurement.

2.3. Receptor analysis and complementation

LT2C-derived mutant strains used for phage receptor analysis are listed in Table S3. Briefly, an overnight culture of various LT2C mutants was mixed with 0.4% molten LB soft agar to generate a bacterial lawn as described above. Phage dilutions were spotted onto the bacterial lawns and incubated. After incubation, the formation of single plaques was examined to determine the phage receptor. To confirm the phage receptor, complement strains for *rfbP, btuB,* and *flgK* genes were further subjected to spotting assays. Primers used for mutant construction and gene complementation are listed in Supplementary Table S4.

2.4. Bacteriophage host range determination

Bacterial strains used for phage host range analysis are listed in Table 1. Each strain was incubated overnight at 37 °C with shaking and then 100 μL of each bacterial culture was added to 5 mL of 0.4% molten LB soft agar and mixed. The mixture was then overlaid on a 1.5% LB agar plate and dried for 20 min at room temperature. Subsequently, serially 10-fold diluted phage lysates were spotted onto the prepared bacterial lawns and incubated at 37 °C for 12 h. After incubation, formation of single plaques was recorded to determine the sensitivity of each bacterium. The efficiency of phage infectivity (EOP) of each strain was compared to that of the LT2C host strain.

2.5. Transmission electron microscopy (TEM) analysis

The morphology of the phages was investigated by transmission electron microscopy (TEM). Each phage stock dilution (approximately 1×10^9 to 1×10^{10} PFU/mL) was placed on a carbon-coated copper grid and negatively stained with aqueous 2% uranyl-acetate (pH 4.0) for 30 s. Phage samples were examined using an energy-filtering transmission electron microscope (LIBRA 120, Carl Zeiss) operated at 80 kV at the National Academy of Agricultural Science (Suwon, South Korea). Phages were identified and classified according to the guidelines of the International Committee on Taxonomy of Viruses (Rodhain, 1995).

Table 1
Host range analysis of the three phages.

Bacterial strain	BSPM4	BSP101	BSP22A
***Samonella enterica* serovar Typhimurium**			
SL1344	+ +	+	+
UK1	+ + +	+	+ + +
LT2	+	+	+
LT2C	+	+	+
ATCC 14028	+	+	+
ATCC 19586	+ +	+	+
ATCC 43147	+ + +	+	+ +
ATCC 13076	+	+	+
DT104	+	+	+
serovar Typhi Ty 2-b	–	–	+
serovar Parathyphi			
A IB 211	–	+	+
B IB 231	–	+	+
serovar Dublin IB 2973	–	+	–
Escherichia coli			
MG1655	–	–	+
***Escherichia coli* O157:H7**			
ATCC 35150	–	+	–
ATCC 43890	–	+	–
ATCC 43894	–	+ +	–
ATCC 43895	–	+ +	–
O157:NM 3204-92	–	+ +	–
O157:NM H-0482	–	+ +	–
Gram-negative bacteria			
Shigella flexineri 2a strain 2457T	–	+	+
Shigella boydii IB 2474	–	–	–
Vibrio fischeri ES-114 ATCC 700601	–	–	–
Pseudomonas aeruginosa ATCC 27853	–	–	–
Cronobacter Sakazakii ATCC 29544	–	–	–
Gram-positive bacteria			
Listeria monocytogenes ATCC 19114	–	–	–
Staphylococcus aureus ATCC 29213	–	–	–
Staphylococcus epidermis ATCC 35983	–	–	–
Bacillus subtilis ATCC 23857	–	–	–
Bacillus cereus ATCC 14579	–	–	–

*, + + +, EOP of 2 to 1.5; + +, EOP of 1.5 to 1; +, EOP of 1 to 0.5; −, not susceptible to phages.
†, ATCC, American Type Culture Collection.

2.6. Extraction of bacteriophage genomic DNA

Bacteriophage genomic DNA was purified from concentrated phage high titer stock (about 10^9 to 10^{10}) as described previously (Wilcox et al., 1996). Prior to extracting phage DNA, phage lysates were treated with DNase and RNaseA at 37 °C for 30 min to remove non-phage DNA or RNA contaminants. Then, 20 mM of EDTA (pH 8.0), 50 μg/mL of proteinase K, and 0.5% of sodium dodecyl sulfate (SDS) were added and the phage lysate was incubated for 15 min at 65 °C. Finally, standard phenol-chloroform DNA purification with ethanol precipitation was carried out to obtain purified phage genomic DNA.

2.7. Genome sequencing and bioinformatics analysis

Extracted phage genomic DNA was sequenced using a Genome Sequencer FLX titanium sequencer (Roche, Mannheim, Germany) and assembled with GS *de novo* assembler software (Roche) at Macrogen Inc., South Korea. Prediction of open reading frames (ORFs) was performed using the combination of FgenesB (Softberry, Inc., Mount Kisco, NY, USA), Glimmer v3.02 (Delcher et al., 2007), and GeneMarkS (Lukashin and Borodovsky, 1998) software. Predicted ORFs were annotated based on the results of InterProScan (Ziedaite et al., 2005) and BLASTP (Altschul et al., 1997) analysis. For comparative analysis of receptor-related genes, genomes of phages with known receptors were used (Table 2).

2.8. Bacterial challenge assay

Fifty milliliters of LB broth was inoculated with 500 μL of an overnight culture of *S.* Typhimurium LT2C, and these cultures were incubated at 37 °C with shaking (220 rpm) until early exponential phase growth (OD_{600} = 0.3–0.4). At that time, cultures were infected with each phage or the cocktail of three phages at an MOI of 1 (approximately 1×10^8 PFU). Addition of SM buffer only to the bacterial culture served as the negative control. After phage infection, bacterial samples were collected every 1 h and the OD_{600} was measured. Bacterial samples were collected at indicated time points and plated on LB agar medium to count the number of viable cells. Samples were diluted 10-fold if necessary. All experiments were performed in triplicate.

2.9. Motility assay

The motility of bacteriophage-insensitive mutants (BIMs) obtained after three-phage cocktail treatment was examined. Briefly, 1 μL of the overnight culture of resistant strain was injected onto solidified soft-agar motility plate (LB medium containing 0.3% agar) and incubated at 37 °C for 5 h. *S.* Typhimurium LT2C was used as a positive control.

2.10. Frequency of bacteriophage-insensitive mutants

Frequency of BIM emergence was determined as described previously (Garcia et al., 2007). Briefly, an appropriate volume of an overnight culture of *S.* Typhimurium LT2C (10^8 CFU/mL) was mixed with each single phage, with two-phage cocktails, or with a cocktail of all three phages at an MOI of 100. After a 10-min incubation at 37 °C, mixtures were serially diluted in sterile Butterfield's phosphate-buffered dilution water and plated on LB agar. These plates were then incubated at 37 °C for 12 h. BIM frequency was calculated by dividing the number of surviving cells by the initial cell number. All experiments were performed in triplicate. The BIMs obtained for each single phage were sub-cultured and subjected to susceptibility tests with the other two phages. The statistical significance of the results was confirmed at the 5% level using a one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests.

2.11. Food application

Fresh iceberg lettuce and cucumber were purchased from the local market and stored at 4 °C before use. For bacterial strain preparation, the host strain was incubated in LB broth at 37 °C with shaking (220 rpm) until the OD_{600} value reached 0.3 (1×10^8 CFU/mL). One-milliliter of cell culture was centrifuged at 13000 × g for 2 min to remove the LB broth and then resuspended in 1 mL of sterile Butterfield's phosphate-buffered dilution water. The prepared cells were serially diluted (10-fold) and used to artificially contaminate food samples as described previously (Kim and Ryu, 2014). Briefly, the inner leaves of iceberg lettuce (*Lactuca sativa*) were cut and sliced into 2 × 5 cm pieces (approximately 10 cm^2) after removing the outer leaves. Cucumber (*Cucumis sativus*) was cut into slices with an average diameter of 3.6 cm (approximately 10 cm^2) using a slicer after washing the surface with 70% ethanol and allowing it to air dry. Both sides of the sliced produce samples were UV-treated for at least 1 h in a bio-safety cabinet to decontaminate indigenous bacteria or phages. Then 100 μL of prepared *Salmonella* inoculum (1×10^6 CFU/mL) was spotted onto each food sample. After 1 h of drying at room temperature (25 °C) in the biosafety cabinet, 1 mL of prepared phage cocktails with different MOI values (10^3 to 10^4) were pipetted onto the surface of each sample and incubated at room temperature (25 °C). For the non-phage treated control group, the same volume of SM buffer used. At the indicated time points, treated samples were transferred into sterile stomacher bags (Labplas Inc., Sainte-Julie, Quebec, Canada) containing 90 mL of buffered peptone water (BPW) and homogenized for 2 min with a BagMixer 400

Table 2
Comparative analysis of genes related to host receptors.

ORF	Predicted function	Length[a]	% identity[b]	BLASTP matches	Receptor[c]	Morphology	Reference
BSPM4 (*Siphoviridae*)							
BSPM4_ORF31	Tail fiber protein	246	98% over 246 aa	Hypothetical protein (ORF_043) from *Salmonella* phage iEPS	Flagella	*Siphoviridae*	(Choi et al., 2013)
			98% over 246 aa	Tail fiber protein from *Salmonella* phage Chi	Flagella	*Siphoviridae*	(Lee et al., 2013)
			99% over 221 aa	hypothetical protein (ORF_043) from *Salmonella* phage SPN19	Flagella	*Siphoviridae*	(Shin et al., 2012)
BSP101 (*Myoviridae*)							
BSP101_ORF81	Tail spike protein	698	99% over 698 aa	Tail spike protein (ORF_0162) from *Salmonella* phage SFP10	O-antigen	*Myoviridae*	(Park et al., 2012)
			80% over 619 aa	Tail spike protein (ORF_055) from *Salmonella* phage 9NA	O-antigen	*Siphoviridae*	(Andres et al., 2012)
			51% over 709 aa	Tail spike protein (ORF_708) from *Salmonella* phage Det7	O-antigen	*Myoviridae*	(Walter et al., 2008)
			36% over 551 aa	Tail spike protein (Gene 9) from *Salmonella* phage P22	O-antigen	*Podoviridae*	(Vander Byl and Kropinski, 2000)
BSP22A (*Siphoviridae*)							
BSP22A_0169	Receptor-binding protein	593	83% over 595 aa	Receptor-binding protein (ORF_0143) from *Salmonella* phage SPC35	BtuB	*Siphoviridae*	(Kim and Ryu, 2012)
			76% over 595 aa	Receptor-binding protein from *Escherichia* phage BF23	BtuB	*Siphoviridae*	(Mondigler et al., 2006)

[a] Number of amino acids.
[b] Amino acid (aa) sequence identity.
[c] Identified phage receptors.

Laboratory Blender (Bagmixer, Interscience). After homogenization, serially diluted sample or 1 mL of undiluted sample was spread-plated using the traditional overlay method (pouring xylose lysine desoxycholate agar on top of resuscitated cells on LB agar 3–4 h after incubation) as described previously (Kang and Fung, 2000). At time points when low cell levels were expected, 5 mL of undiluted samples was equally distributed onto 5 plates of each medium type and plate counting was performed as described previously (Kim et al., 2012). The absence of contamination with other bacteria was confirmed in the non-inoculated samples using the same methods as used in the experimental samples. All tests were conducted in triplicate.

3. Results and discussion

3.1. Selection of S. Typhimurium-infecting phages based on host receptors and host ranges

A phage cocktail consisting of phages targeting different receptors could potentially be effective at delaying the development of resistant strains (Yen et al., 2017). As a prerequisite to constructing an effective phage cocktail, *S*. Typhimurium-infecting phages targeting different receptors were isolated from various environmental samples by screening with a wild-type strain and its isogenic mutants defective in *rfaC, rfbP, btuB* and *flgK* in the form of single, double, and triple mutations (*ΔrfaC*, *ΔrfaCbtuB* and *ΔbtuBrfbPflgK*) (Tables S2 and S3). These mutants were chosen as host strains for phage screening to isolate the phages targeting other receptors except for flagella, the O-antigen of LPS, and the outer membrane protein BtuB, because they were already isolated by our group using the wild type *S*. Typhimurium strain as a host (Shin et al., 2012). The 21 phages newly isolated in this study utilize five different receptors, namely the flagella, O-antigen, BtuB, core oligosaccharide (OS) region of LPS, and the outer membrane protein OmpC (Table S3). Consistent with our previous study (Shin et al., 2012), phages targeting the flagella, O-antigen, and BtuB were the most frequently isolated (Table S2).

Phage cocktails can also solve the problem of the narrow host range of phages. We therefore considered the host range of each phage for phage selection to develop a broad host-range phage cocktail (Table 1). Among the O-antigen-targeting phages, BSP101, which was isolated from the swine intestine, inhibited host growth most efficiently (Fig. 1A). Among the O-antigen-targeting phages, only the phage BSP101 formed clear plaques, suggesting that this phage has a virulent phenotype (Tables S2 and S3). Phages BSPM4 (flagella targeting) and BSP22A (BtuB targeting) inhibited host growth better than other phages that targeted the same receptors (Fig. 1B and C). Phages BSPM4 and BSP22A were isolated from soil and pond water, respectively (Table S2). They also formed clear plaques, which indicates virulent phenotypes. In addition, host range tests of three phages (BSP101, BSPM4, and BSP22A) revealed that these three phages infected all tested *S*. Typhimurium strains and formed clear plaques (Table 1). Host ranges of the three phages were completely complementary. Therefore, a phage cocktail containing these three phages was able to infect all tested *Salmonella* strains. Phage BSP101 infected *E. coli* O157:H7 strains and BSP22A infected other *Salmonella* serovars, including *Salmonella* Typhi, *Salmonella* Paratyphi, *Salmonella* Dublin, and *Shigella flexneri* (Table 1). These results indicate that a phage cocktail containing BSPM4, BSP101, and BSP22A may inhibit pathogenic bacteria other than *Salmonella* such as *E. coli* O157:H7 and *S. flexneri*, which are important foodborne and waterborne pathogens. Most recently-reported phage cocktails targeting *Salmonella* have a broader host range than single phages, but their infection range is limited to *Salmonella* species only (Bao et al., 2015; Bardina et al., 2012; Hooton et al., 2011).

Phages targeting the core oligosaccharide (OS) region (BSP3 and BSP16), BtuB (BSPM1, BSPM2 and BSPM3), and OmpC (BSP25) were not able to infect the wild type *Salmonella* strain, which has an intact LPS structure with O-antigen (Table S3). A previous study showed that a rough type host bacteria-targeting phage SSU5 could delay the emergence of resistant strains when treated with the two O-antigen-specific phages SSU14 and P22, respectively (Kim et al., 2014). However, in this study, two phages (BSP3 and BSP16) targeting the core OS region did not delay the development of phage-resistant *Salmonella* strains when used in combination with the phage BSP101. This may be because treatment with the phage BSP101 did not generate O-antigen deficient mutants, while treatment with the two temperate phages SSU14 and P22 did (Kim et al., 2014). Indeed, mutant strains derived from BSP101 phage infection were not sensitive to other rough type-specific phages (BSPM1, BSPM2, BSPM3, and BSP25) isolated in this study (data not shown). Because none of the rough type *Salmonella*-specific phages isolated in this study (BSP3, BSP16, BSPM1, BSPM2, BSPM3, and BSP25) had a synergistic effect on host growth inhibition

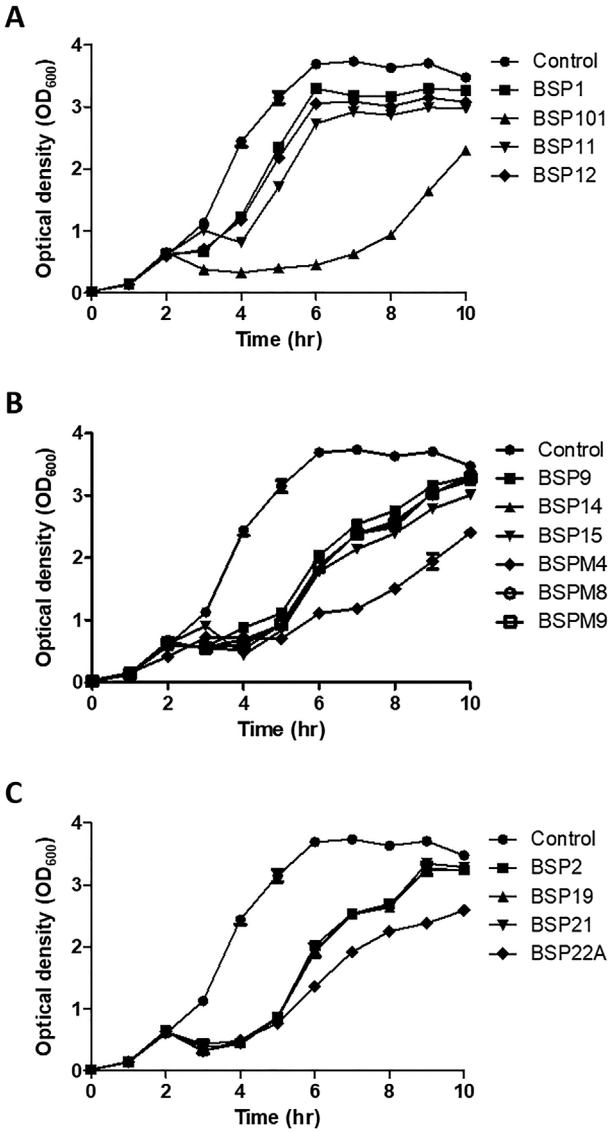

Fig. 1. Inhibition assays of LT2C with each phage. A, O-antigen-targeting phages; B, Flagella-targeting phages; C, BtuB targeting phages. ●, control group without phage; ■, ▲, ▼, ◆, O, □, each group was treated with a phage MOI of 1.

when applied together with O-antigen targeting phage, we exclude those six phages from the phage cocktail. An OmpC-targeting phage, BSP64, was found to be a lysogenic temperate phage. Therefore, this phage was excluded from further study because only virulent phages that have strong host growth inhibition activity without lysogen formation are generally considered to be suitable biocontrol agents.

Three *S.* Typhimurium phages that effectively inhibited growth (Fig. 1), formed clear plaques (Fig. 2), and showed complementary host ranges (Table 1) were selected from among phages targeting three major receptors (flagella, O-antigen, and BtuB) for further experiments. The receptors of phages BSPM4, BSP101, and BSP22A were confirmed to be flagella, O-antigen, and BtuB, respectively, based on mutants lacking these three genes and complementation tests (Fig. 2). Further analysis using BIM strains obtained after each phage treatment revealed that BIM strains appeared by one phage treatment were infected by two other phages (Fig. 3), indicating that no cross-resistance developed in *S.* Typhimurium against these phages. Receptor analysis and the BIMs susceptibility test suggest that the three phages selected can independently infect the host using its own receptor regardless of the development of bacterial resistance against the other phages (Gu et al., 2012).

3.2. Morphological analysis of BSPM4, BSP101, and BSP22A

TEM analysis of phages revealed that BSPM4 and BSP22A belong to the *Siphoviridae* family (Fig. 4A and B). BSPM4 has a long, non-contracted tail of 229 ± 5 nm length (n = 3) with coiled tail fibers, and its head size is 71 ± 2 nm (n = 3). BSP22A has a non-contracted tail (179 ± 5 nm, n = 3) without a distinct tail fiber structure and an icosahedral capsid (95 ± 2 nm, n = 3). In general, the size of a phage capsid corresponds to its genome size (Petrov et al., 2010). Therefore, we predicted that phage BSP22A would have a larger genome than phage BSPM4. BSP101 has a contracted tail (123 ± 5 nm, n = 3) with short tail spikes and a big head (97 ± 4 nm, n = 3), a morphology typical of *Myoviridae* phages (Fig. 4C) (Aksyuk et al., 2011). TEM analysis indicated that the three phages were morphologically different from each other. These morphological differences suggest distinct host recognition mechanisms during host infection.

3.3. Analysis of the genomes of BSPM4, BSP101, and BSP22A

Virulent phages are generally considered to be suitable for the development of phage-based biocontrol agents because they are unable to integrate into the host genome (Hagens and Loessner, 2007). To examine the genomic characteristics of the phages, whole genome sequencing was performed; the Genbank accession numbers for phages BSPM4, BSP101, and BSP22 are KY620117, KY787213, and KY787212, respectively. Bioinformatics analysis revealed that phages BSPM4, BSP101, and BSP22A have 78, 219, and 174 open reading frames (ORFs), respectively. In general, phages that form clear plaques are considered to be virulent phages (Bao et al., 2015; Li and Zhang, 2014; Mirzaei and Nilsson, 2015). Because all three phages formed clear plaques (Fig. 2), we expected them to be virulent phages. Consistent with this expectation, genes related to phage lysogen decision (integrases, excisionase, or repressors) were not identified in any of the three phage genomes (Fig. S1), confirming that these are virulent phages (Chang et al., 2015). Moreover, genes related to virulence, toxins, or drug resistance were not discovered in the genomes of the

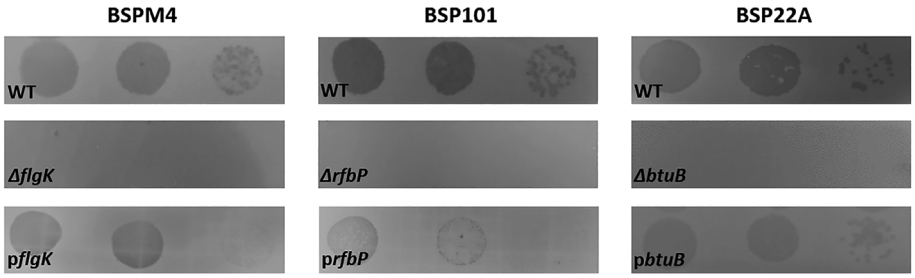

Fig. 2. Identification of S. Typhimurium host receptors. Receptor gene complementation of the *flgK* gene for BSPM4, *rfbP* gene for BSP101, and *btuB* gene for BSP22A. Ten-fold serially diluted phage samples were dotted in each chamber.

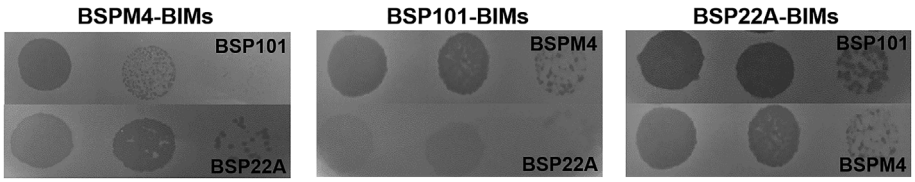

Fig. 3. Susceptibility of BIMs to other receptor-targeting phages. Ability of the three phages to infect BIMs obtained after other two phage treatments. All experiments were conducted in triplicate.

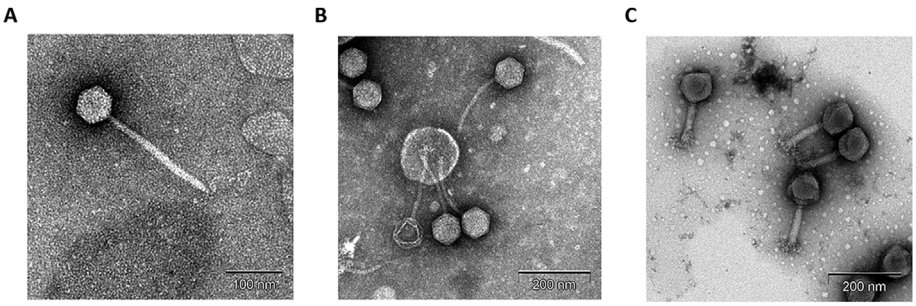

Fig. 4. TEM images of the isolated phages. (A) BSPM4, (B) BSP22A, and (C) BSP101.

three phages (Fig. S1) (Cui et al., 2017).

Genome sizes of BSPM4, BSP101, and BSP22A are 59,097 bp, 157,665 bp, and 110,741 bp, respectively, and G + C% contents are 56.5%, 44.5%, and 40.01%, respectively (Fig. S1). Although both BSPM4 and BSP22A are *Siphoviridae* phages with a long flexible tail (Fig. 4A and B), the genome of BSP22A is almost two-fold larger than that of BSPM4 (Fig. S1). Whole genome sequence analysis indicated that BSPM4 and BSP22A are Chi-like and T5-like phages, respectively. The large genome of BSP101 (ca. 158 Kbp) (Fig. S1) is consistent with it being a *Myovirus* phage as these phages usually have genomes larger than 125 Kbp (Hatfull, 2008), supporting the TEM analysis results (Fig. 4C). Whole genome sequence analysis confirmed that BSP101 belongs to the Vi01 phage-like family *Myoviridae*.

Genes associated with host recognition were identified in each phage genome, including a tail fiber protein for BSPM4, a tail spike protein for BSP101, and a receptor-binding protein for BSP22A, respectively (Table 2). Comparative genomic analysis revealed that those proteins have high homology to the corresponding proteins in previously-reported phages (Table 2), but no homologies were observed among the receptor recognition genes of the three phages. The tail fiber protein (ORF_31) of BSPM4 showed 98–99% amino acid identity to hypothetical or tail fiber proteins of flagella-targeting phages such as iEPS5, Chi, and SPN19, indicating that this tail fiber protein of phage BSPM4 is important for recognizing host flagella (Table 2). Among four tail-related genes in BSP101, ORF_81 showed the high amino acid identity of 99% with the tail spike gene of O-antigen-targeting SFP10 (ORF_162), which is responsible for O-antigen recognition (Park et al., 2012). This suggests that BSP101 recognizes the O-antigen. Surprisingly, the tail spike protein of BSP101 also showed high amino acid homology (80%) with that of *Siphoviridae* phage 9NA (Table 2). The homology (36%–99%) among tail spike proteins of *Myoviridae* phages (BSP101 and SFP10), a *Siphoviridae* phage (9NA), and a *Podoviridae* phage (P22) suggests that these proteins may be widely distributed among O-antigen-targeting phages (Walter et al., 2008), regardless of phage morphology. Moreover, all four tail spike proteins contain a phage P22 tail domain (pfam09251), indicating functional involvement in O-antigen recognition (Table 2). Phage BSP22A has a receptor-binding protein with amino acid homologies of 76–83% to receptor-binding proteins of other BtuB-targeting phages, lending support to the hypothesis that BtuB is the receptor targeted by BSP22A (Table 2). The comparative genome analysis revealed that genes important for receptor recognition are highly conserved in phages that utilize the same receptors.

Interestingly, genes related to superinfection immunity were identified in the genomes of BSP101 (ORF_146, superinfection exclusion protein) and BSP22A (ORF_170, receptor blocking protein), while BSPM4 has no genes for superinfection immunity (Fig. S1). Usually, superinfection immunity is against the same phage group or a closely related phage group (Bergua et al., 2014; Labrie et al., 2010). TEM analysis and whole genome sequencing results revealed that the three phages belong to different phage groups (Chi-like, T5-like, and Vi01-like, respectively), and no nucleotide homologies were observed among the three phage genomes (Fig. S1 and Fig. 4). These results suggested that the three phages are free from superinfection exclusion by other phages. Taken together, genome analyses suggested that the three different phages would be good candidates for the development of a phage cocktail.

3.4. Bacterial challenge assay

The efficiency of the phage cocktail was evaluated using the *in vitro* bacterial challenge assays. Whereas BSPM4 and BSP101 significantly inhibited the growth of *Salmonella* ($p < 0.05$) for a few hours after infection, BSP22A only slightly reduced bacterial growth for several hours after infection (Fig. 5A). Combinations of two phages (i.e., BSPM4 & BSP101, BSPM4 & BSP22A, and BSP101 & BSP22A) inhibited *Salmonella* to the same extent as single treatment with BSPM4 or BSP101 (Fig. 5A). Simultaneous treatment of *Salmonella* with all three phages resulted in efficient lysis and inhibition of growth for 6 h (Fig. 5A), and there was a significant reduction in the number of viable cells ($p < 0.05$) compared to single and double-phage treatments after 12 h (Fig. 5B). These results are similar to those reported previously for *Vibrio cholera*-targeting phages, where a phage cocktail treatment achieved lower cell density than single phage treatment (Yen et al., 2017). Therefore, simultaneous treatment with three phages targeting three different receptors resulted in substantially higher inhibition of host cell growth than treatment with one or two phages. Moreover, loss of motility was observed in bacteriophage-insensitive mutants (BIMs) that emerged after treatment with the three-phage cocktail (Fig. 5C). This is likely a resistance mechanism to escape from infection by the flagella-targeting phage BSPM4, as most bacteria resistant to flagella-targeting phages tend to lose motility (Evans et al., 2010). Because motility is an important virulence factor in *Salmonella* (Methner and Barrow, 1997), bacteria resistant to the phage cocktail treatment would have compromised virulence (Leon and Bastias, 2015). These results suggest that simultaneous treatment with three phages targeting different receptors is a promising strategy to control *S*. Typhimurium by reducing both bacterial number and virulence.

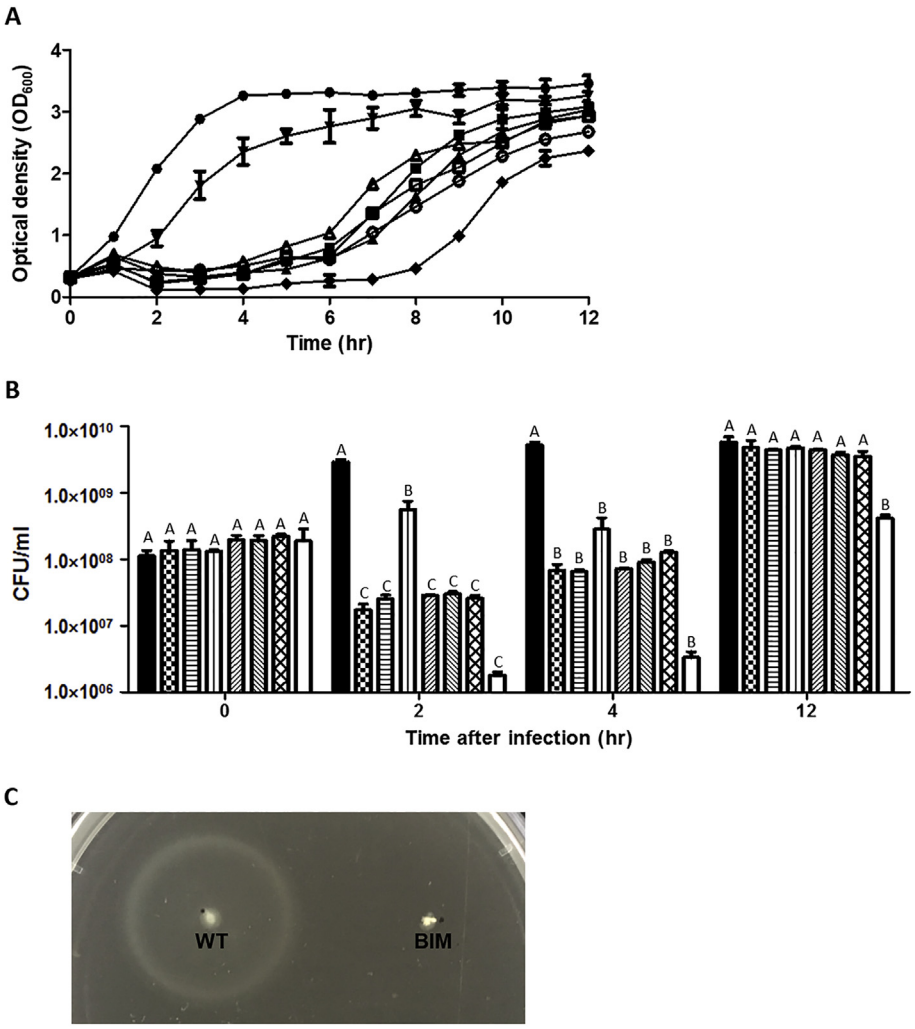

Fig. 5. Challenge test of *S*. Typhimurium LT2C with phages BSPM4, BSP101, BSP22A and a cocktail of the three phages. (A) Graphs showing OD values of samples after phage infection. Closed circles, non-phage-infected sample; closed squares, BSPM4-infected sample; closed triangles, BSP101-infected sample; reverse triangles, BSP22A-infected sample; open circles, BSPM4 and BSP101-infected sample; open squares, BSPM4 and BSP22A-infected sample; open triangles, BSP101 and BSP22A-infected sample and closed diamonds, three-phage cocktail-infected sample. (B) Numbers of viable cells were determined at the indicated time points; non-phage-infected sample (■), BSPM4-infected sample (▣), BSP101-infected sample (▤), BSP22A-infected sample (▥), BSPM4 and BSP101-infected sample (▨), BSPM4 and BSP22A-infected sample (▧), BSP101 and BSP22A-infected sample (▩), and three-phage cocktail-infected sample (□). Each column represents the mean of triplicate experiments, and error bars indicate the standard deviation. One-way analysis of variance (ANOVA) with Duncan's multiple range tests were used at each time point. Capital letters indicate significant differences ($p < 0.05$) between test groups. (C) Motility of the WT LT2C strain and BIMs obtained after three-phage cocktail treatment.

3.5. Frequencies of BIM development and phage susceptibility of BIMs

Because the three-phage cocktail extended the period of growth inhibition of *Salmonella* and delayed the emergence of resistant strains, we investigated the effect of the three-phage cocktail treatment on the emergence of bacteriophage-insensitive mutants (BIMs) (Garcia et al., 2007). The frequencies of BIM occurrence were significantly lower in two double-phage cocktails (i.e., BSPM4 & BSP101 and BSP101 & BSP22A) and the three-phage cocktail than when single phages were used (Table 3). Cocktails containing BSP101 significantly inhibited resistance development (Table 3). To date, cross-resistance between flagella-targeting phages and O-antigen-targeting phages has not been discovered. Our results indicated that simultaneous application of phages targeting flagella and O-antigen efficiently reduced the development of resistant strains (Table 3).

In addition, co-treatment with phages targeting BtuB and O-antigens (BSP22A and BSP101, respectively) also effectively reduced the emergence of resistant strains. Variations in O-antigen biosynthesis or modifications can confer resistance to infection by phages targeting the O-antigen and BtuB (Knirel et al., 2015; Shin et al., 2012). As described above, O-antigen glucosylation is a resistance mechanism against BtuB-targeting phages in *S*. Typhimurium (Kim and Ryu, 2012). Indeed, continuous O-antigen glucosylation of the host strain by a plasmid expressing *gtrABC1* genes inhibited infection by BSP22A (BtuB phage). However, the *gtrABC1* gene-expressing strain remained susceptible to BSP101 (O-antigen phage) infection (data not shown). This result highlights the effectiveness of using phages targeting two different receptors. Moreover, BIM strains derived from treatment with phages BSP101 and BSP22A, respectively, were susceptible to subsequent infection by the other phage (BSP22A and BSP101, respectively), further demonstrating how co-treatment with two phages can reduce the development of bacterial resistance (Fig. 3). Another possibility is that *Salmonella* may develop an O-antigen-deficient mutation that is not a transient resistance mutation (Kim et al., 2014). However, this type of resistance is disadvantageous to the host, because an O-antigen-deficient mutant can be infected by BtuB-targeting phages, including BSP22A (Table S3). Therefore, we speculate that development of bacterial resistance against both phages may affect bacterial growth. Although the exact resistance mechanism remains unknown, our data

Table 3
Determinations of the frequencies of BIMs.

Phage treatment	BIM frequency (mean ± SD[a])	Fold-change[b]
BSPM4	$1.63 \times 10^{-3} \pm 2.63 \times 10^{-4}$ C	8.25
BSP101	$1.11 \times 10^{-3} \pm 2.08 \times 10^{-4}$ CD	5.64
BSP22A	$9.75 \times 10^{-3} \pm 6.73 \times 10^{-4}$ A	49.5
BSPM4 + BSP101	$6.28 \times 10^{-4} \pm 3.95 \times 10^{-5}$ DE	3.20
BSPM4 + BSP22A	$5.92 \times 10^{-3} \pm 6.12 \times 10^{-4}$ B	30.0
BSP101 + BSP22A	$7.40 \times 10^{-4} \pm 7.71 \times 10^{-5}$ DE	3.80
BSPM4 + BSP101 + BSP22A	$1.97 \times 10^{-4} \pm 9.34 \times 10^{-5}$ E	1.00

Capital letters indicate significant differences ($p < 0.05$) between test groups.
[a] SD, standard deviation. Results were obtained from triplicate experiments.
[b] Fold-change values are relative to the three-phage cocktail treatment result.

showed that co-treatment of *S*. Typhimurium with BSP101 and BSP22A reduced the occurrence of BIMs compared to each phage treatment alone. Dual treatment with BSPM4 and BSP22A significantly reduced resistance development by 1.7-fold compared to single treatment with BSP22A (Table 3). It appears that the two phages had an additive effect on reducing the development of bacterial resistance, presumably because the resistance mechanisms against the flagella-targeting phage BSPM4 and the BtuB-targeting phage BSP22A are independent of each other, as observed previously (Shin et al., 2012).

The three-phage cocktail reduced the development of BIMs 3.20-fold compared to double-phage cocktail treatment with BSPM4 & BSP101, and 49.5-fold compared to single phage (BSP22A) treatment (Table 3). Differences in BIM frequency between phage cocktail treatments and single phage treatments have not been observed in previous studies in which bacteriophage receptors were not identified (O'Flynn et al., 2006; O'Flynn et al., 2004). These results indicated that treatment using three phages that target different receptors may significantly reduce the development of resistance against phage infection in *Salmonella*.

The results of the BIM testing clearly showed that simultaneous treatment with different receptor-targeting phages in a cocktail successfully reduced the development of bacterial resistance to phage infection. In addition, we obtained new insights into the combinatorial effects of simultaneously treating *Salmonella* with flagella, O-antigen, and BtuB-targeting phages.

3.6. Efficacy validation of the three-phage cocktail in fresh produce spiked with Salmonella

The efficiency of the phage cocktail treatment to control *Salmonella* contamination in fresh produce was evaluated using iceberg lettuce and cucumber as model systems. The food samples were washed with 70% ethanol and UV-treated to decontaminate indigenous bacteria or phages. We tried to eliminate the unpredictable background effects caused by indigenous bacteria or phage of food samples because variations in microflora of food samples would affect phage cocktail treatment differently even though the influence of background microflora should not be high considering the narrow host specificity of phages. Treatment of fresh produce samples spiked with *S*. Typhimurium LT2C with phage cocktail at an MOI of 10^3 resulted in a significant reduction in bacterial growth ($p < 0.05$) on iceberg lettuce from 1.1 to 1.9 log CFU/cm^2 (Fig. 6A) and on cucumber from about 0.7 to 1.2 log CFU/cm^2 (Fig. 6B) after 4 h of treatment at 25 °C. When fresh produce was treated with phages at an MOI of 10^4, a significant reduction in bacterial growth was achieved ($p < 0.05$); 2.8 to 3.9 log CFU/cm^2 reduction on iceberg lettuce (Fig. 6A) and 2.5 to 2.8 log CFU/cm^2 reduction on cucumber (Fig. 6B) after 4 h at 25 °C. In a previous study, treatment of *S.* Enteritidis-contaminated Chinese cabbage by a cocktail of two phages reduced growth by 3.0 log CFU/cm^2 after 5 h at 25 °C (Bao et al., 2015). Research by another group demonstrated that a phage cocktail containing four lytic phages reduced *S.* Enteritidis populations on fresh-cut honeydew melons by 2.5 log CFU at room temperature (20 °C) (Leverentz et al., 2001). Treatment of cucumbers artificially contaminated with *Salmonella* Newport with the commercial six-phage cocktail SalmoFresh decreased bacterial populations by 1.83 log in a day during storage at 4 °C (Sharma et al., 2017).

In our study, a cocktail of three phages achieved a 3.9 log reduction of *S*. Typhimurium numbers on lettuce and a 2.8 log reduction on cucumber after a 4 h incubation at 25 °C (Fig. 6), indicating strong bactericidal effect of our phage cocktail against *S*. Typhimurium on fresh produce. In addition, the bacterial killing effect was sustained for more than 12 h in both lettuce and cucumber with further viability reductions of 5.5–5.8 log CFU after treatment with an MOI of 10^4 phages. Rise of the phage titer (PFU/cm^2) of the food samples was observed after 12 h of incubation (data not shown), suggesting that the development of bacterial resistance against the three-phage cocktail in our model system is low probably because the three phages use different receptors (Fig. 6). The three-phage cocktail therefore holds promise as a novel, natural disinfecting agent that can improve the safety of fresh produce by eradicating *S*. Typhimurium contamination.

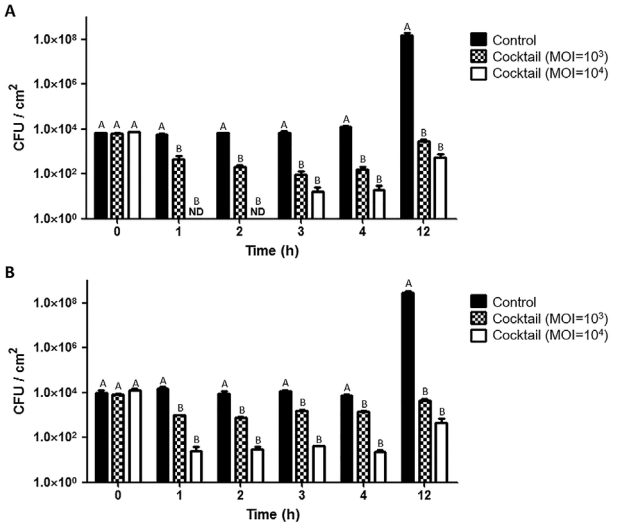

Fig. 6. Food application of the phage cocktail. Control efficiency of the three-phage cocktail was tested against *S*. Typhimurium in (A) iceberg lettuce and (B) cucumber. Numbers of viable cells were determined at the indicated time points; control sample (■), cocktail-treated samples with an MOI of 10^3 (▣), cocktail-treated samples with an MOI of 10^4 (□). Detection limit of the experiment was 1.0 log CFU. Mean values from two independent measurements are plotted. One-way analysis of variance (ANOVA) followed by Duncan's multiple range tests were used at each time point. Capital letters indicate significant differences ($p < 0.05$) between test groups. ND, not detected.

4. Conclusions

Outbreaks of *S*. Typhimurium caused infections due to contamination of food are frequent and affect humans all over the world. We therefore developed a novel phage cocktail targeting *Salmonella*. We isolated and characterized three novel phages targeting different receptors (i.e., flagella, O-antigen and BtuB) of *S*. Typhimurium. BIMs susceptibility test revealed that no cross-resistances were observed among those phages. Genomic study of the three phages revealed that all three phages are strictly virulent and do not have toxin or virulence related genes in their genomes, suggesting they are safe for application to foods (McCallin et al., 2013). *In vitro* challenge assays and BIM frequency analysis revealed that the three-phage cocktail inhibited host growth to a greater extent than two phage cocktails and single phages, in addition to decreasing the occurrence of resistant strains, indicating that preparation of a cocktail consisting of phages utilizing different host targets is a powerful strategy to control *S*. Typhimurium. The strategy for phage cocktail preparation based on receptor utilization described in this study can be applied to develop phage cocktails against other pathogens. Furthermore, effective inhibition of *S*. Typhimurium in fresh produce by the phage cocktail underscores the potential of this phage cocktail as a biocontrol agent against *S*. Typhimurium contamination of fresh produce.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fm.2018.08.011.

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