Characterization of a T5-Like Coliphage, SPC35, and Differential Development of Resistance to SPC35 in *Salmonella enterica* Serovar Typhimurium and *Escherichia coli*[∇][†]

Minsik Kim and Sangryeol Ryu*

Laboratory of Molecular Food Microbiology, Department of Food and Animal Biotechnology, Department of Agricultural Biotechnology, Research Institute for Agriculture and Life Sciences, and Center for Agricultural Biomaterials, Seoul National University, Seoul 151-921, South Korea

The potential of bacteriophage as an alternative biocontrol agent has recently been revisited due to the widespread occurrence of antibiotic-resistant bacteria. We isolated a virulent bacteriophage, SPC35, that can infect both *Salmonella enterica* serovar Typhimurium and *Escherichia coli*. Morphological analysis by transmission electron microscopy and analysis of its 118,351-bp genome revealed that SPC35 is a T5 group phage belonging to the family *Siphoviridae*. BtuB, the outer membrane protein for vitamin B_{12} uptake, was found to be a host receptor for SPC35. Interestingly, resistant mutants of both *E. coli* and *S.* Typhimurium developed faster than our expectation when the cultures were infected with SPC35. Investigation of the *btuB* gene revealed that it was disrupted by the IS*2* insertion sequence element in most of the resistant *E. coli* isolates. In contrast, we could not detect any *btuB* gene mutations in the resistant *S.* Typhimurium isolates; these isolates easily regained sensitivity to SPC35 in its absence, suggesting phase-variable phage resistance/sensitivity. These results indicate that a cocktail of phages that target different receptors on the pathogen should be more effective for successful biocontrol.

Bacteriophages (phages) are viruses that specifically infect bacteria. They exist everywhere that bacteria thrive at an estimated ratio of at least 10 phage particles per bacterial cell (50). Phages are highly specific for a species; therefore, they are able to target a pathogen without disrupting the natural microflora (25, 39). As multidrug-resistant bacteria have become more common, phages have attracted attention as a potential alternative to antibiotics. Their advantages include their lower cost compared to that for the development of new antibiotics (39), ability to replicate in the presence of host bacteria, lack of side effects, and high host specificity. The clinical use of phages, known as phage therapy (39), has been reported for human and animal diseases caused by *Escherichia coli* (5, 13), *Pseudomonas aeruginosa* (3, 51), *Salmonella* spp. (8, 52), and *Staphylococcus aureus* (40).

Recently, the use of phages to prevent food-borne infectious diseases has also been proposed (21, 25, 27). The impact of food-borne illness on public health is considerable and has great economic significance. These diseases still occur, despite dramatic improvements in hygiene and sanitation in food processing. Phages have been used to control food-borne diseases caused by *Salmonella* spp. (20, 41), *Listeria monocytogenes* (12, 23), *Campylobacter jejuni* (20, 28, 53), and *E. coli* O157:H7 (44, 48). The status of generally recognized as safe (GRAS) was recently given to the *L. monocytogenes*-specific phage Listex

P100, which was approved as a food preservative by the U.S. Food and Drug Administration (9). This has motivated many researchers to investigate the development of phages for biocontrol.

Several limitations must be overcome before phages can be successfully used as therapeutic and/or biocontrol agents. The first limitation is the narrow host range of phages. The high host specificity of most phages is a double-edged sword; high specificity is necessary for the specific targeting of unwanted bacteria, but the narrow host range renders phage biocontrol ineffective for non-host strains (22). The use of broad-host-range phages isolated by multiple-host enrichment methods (7, 11, 29) or a phage cocktail composed of multiple phage types (33, 44, 55) has shown promise in overcoming this problem. Another limitation is the rapid emergence of phage-resistant mutants (6, 11, 18, 32). A phage cocktail can control or delay the emergence of resistant mutants (27, 33); however, some of the underlying mechanisms for the appearance of phage-resistant mutants are not fully elucidated.

In this context, we isolated and characterized the novel lytic bacteriophage SPC35. This bacteriophage has a broad host range that includes the genus *Salmonella* and the genus *Escherichia*, which are important food-borne pathogens of humans and animals (10, 43). Bacterial challenge assays revealed distinctive patterns of growth inhibition and emergence of phage-resistant mutants in these two genera.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. All *Salmonella enterica* serovar Typhimurium and *E. coli* mutants were derived from *S.* Typhimurium SL1344 and *E. coli* MG1655, respectively. *E. coli* isolates were kindly provided by Dong-Hyun Kang at Seoul National University, Seoul, South Korea. Bacteria were cultured at 37°C in

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TABLE 1. Bacterial strains and SPC35 host range

Strain (genotype)	Plaque formation[a]	Source or reference[b]
Salmonella Typhimurium		
UK1	CC	Laboratory collection
LT2	CC	Laboratory collection
SL1344	CC	Laboratory collection
Δ*fhuA* (SL1344 Δ*fhuA*)	CC	25
Δ*fepA* (SL1344 Δ*fepA*)	CC	25
Δ*btuB* (SL1344 Δ*btuB*)	−	25
Δ*btuB*(pMS100) (Δ*btuB* harboring pMS100)	CC	This study
14028s	CC	Laboratory collection
ATCC 19586	CC	ATCC
ATCC 43174	CC	ATCC
DT 104	CC	Laboratory collection
Salmonella Enteritidis ATCC 13078	C	ATCC
Salmonella enterica isolates		
3068	CC	Laboratory collection
3605	TT	Laboratory collection
3792	CC	Laboratory collection
4509	CC	Laboratory collection
Escherichia coli		
ATCC 25922	(+)	ATCC
B/1	(+)	*
B/4	−	*
BE4a	−	*
C600	−	*
DH5α	CC	Laboratory collection
DH10B	CC	Laboratory collection
FS575	T	*
GM1	CC	*
GM119	CC	*
HB101	(+)	*
JM109	CC	*
K-12 substrain MC4100	CC	Laboratory collection
K-12 substrain MG1655	CC	Laboratory collection
K-12 substrain W3110	T	*
K-12 2B	−	*
K802	(+)	*
LE392	−	*
RP1	C	*
SY327	CC	*
TGI	CC	*
Escherichia coli isolates		
C-660	CC	*
E-2g	(+)	*
E-2j	−	*
E-7b	−	*
E-34	−	*
WADDL 2701	CC	*
WADDL 2735	−	*
WADDL 2983	−	*
WADDL 2902	−	*
WADDL 3463	C	*
WADDL 3476	(+)	*
WADDL 3502	−	*
WADDL 3811	−	*
WADDL 3977	−	*
WADDL 4036	−	*
WADDL 4064	(+)	*
WADDL 4083	CC	*
WADDL 4190	−	*
WADDL 4241	−	*

[a] CC, a relatively clearer plaque; C, clear plaque; T, turbid plaque; TT, relatively more turbid plaque; (+), growth inhibition zone; −, no plaque.
[b] ATCC, American Type Culture Collection; *, collection of Food Hygiene laboratory at Seoul National University, Seoul, South Korea.

Luria-Bertani (LB) broth and plates supplemented with 25 μg/ml of chloramphenicol where appropriate.

Plasmid construction. The plasmid used in the complementation assay was constructed by cloning the complete *btuB* gene and its promoter region from

Salmonella SL1344 into the low-copy-number plasmid pACYC184. The *btuB* gene and its promoter region were amplified by PCR using primers btuB-CF-2 (5′-TTG TAG GGC ATG CTC AGT GGA TGT-3′) and btuB-CR-2 (5′-ATA CAA GCT TGG TGG GAC GTG GTT-3′). The PCR product was digested with restriction enzymes SphI and HindIII and then inserted into the corresponding restriction sites of plasmid pACYC184. The DNA insertion was verified by sequencing, and the resultant plasmid was named pMS100.

Isolation of bacteriophages. Ten samples of chicken feces and two samples of chicken internal organs were obtained from the Mo-ran traditional market, Seoul, South Korea. The 25-g samples were homogenized in 225 ml sterile Butterfield's phosphate-buffered dilution water (0.25 M KH_2PO_4 adjusted to pH 7.2 with NaOH) for 90 s with a blender (BacMixer 400; Interscience Laboratory Inc., St. Nom, France). The 10-ml samples were then mixed with 50 ml LB broth and incubated at 37°C for 24 h with agitation (220 rpm). After centrifugation of the culture (9,000 × g, 10 min, 4°C) and filtration of the clear supernatant (0.22-μm-pore-size filter; Millipore, Ireland), the filtrate (10 ml) was mixed with 50 ml LB broth and 500 μl overnight culture of *S*. Typhimurium SL1344. This bacterium-phage mixture was incubated at 37°C for 8 h (at 220 rpm), followed by centrifugation and filtration as described above. Tenfold serial dilutions of these filtrates were used in the spotting assay with *S*. Typhimurium SL1344 or *E. coli* MG1655 to confirm the presence of bacteriophages. To isolate and purify the bacteriophages, the overlay assay was carried out as previously described (2). Each plaque showing a unique morphology was picked with a sterile tip, followed by elution with sodium chloride-magnesium sulfate (SM) buffer (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 10 mM $MgSO_4$). This process was repeated at least three times.

Spotting assay and overlay assay. The host bacteria were cultured overnight, and then 100 μl of them was inoculated into 5 ml of soft LB agar (0.4% agar), which had been heated to 42°C in a water bath. After gentle vortexing of this mixture, it was poured into prepared LB agar plates (1.5% agar with 25 μg/ml chloramphenicol, if necessary) and allowed to solidify at room temperature for 30 min to produce bacterial lawns. Then, 10 μl of phage stock dilutions (10-fold serial dilutions in SM buffer) was spotted onto the top agar layer, and the plates were dried at room temperature for 30 min. These plates were incubated overnight at 37°C and inspected on the next day for single plaques or bacterial growth inhibition zones.

The overlay assay was carried out with modifications. Briefly, phage stock dilutions (100 μl) were mixed with the same volume of overnight bacterial culture in 5 ml soft LB agar (0.4% agar), and the mixture was then poured into plates. After the medium was allowed to solidify for 30 min at room temperature, the plates were incubated at 37°C and plaques were examined on the next day.

Propagation of SPC35. Bacteriophage propagation and concentration were carried out according to the methods of Sambrook and Russell (47), with some modifications. Briefly, the lysate of a single plaque of phage SPC35 was added to an *E. coli* MG1655 culture (optical density at 600 nm [OD_{600}] = 0.5 to 0.6), which was then incubated at 37°C for 4 h. The cleared culture with cell debris (host bacteria lysate) was treated with chloroform (1% of final volume), incubated at 37°C for 5 min, and then centrifuged (15,000 × g, 10 min, 4°C). Bacteriophage particles in the filtered supernatant (0.22-μm-pore-size filter) were precipitated with 10% (wt/vol) polyethylene glycol (PEG) 6000 in 1 M NaCl at 4°C for 10 h. After centrifugation (10,000 × g, 15 min, 4°C), precipitated phages were resuspended in SM buffer and separated by CsCl density gradient ultracentrifugation (78,500 × g, 2 h, 4°C). The phage band fraction was separated by puncturing the centrifuge tube bottom with a heated needle and dialyzed against 1 liter of dialysis buffer (50 mM Tris-HCl [pH 8.0], 10 mM NaCl, 10 mM $MgSO_4$) in a Spectra/Por dialysis membrane (molecular weight cutoff, 12,000 to 14,000; Spectrum Laboratories, Inc.). The dialysis buffer was changed after 1 h, and the phage fraction was dialyzed for an additional 1 h before it was transferred into a sterilized glass ampoule. The titers of these concentrated phage stocks were determined by the overlay assay, and the phage stocks were stored at 4°C until further use.

Morphological analysis by TEM. Drops of SPC35 stock dilution (4 μl, approximately 1×10^{10} PFU/ml) were placed on carbon-coated copper grids; after 1 min, the excess phage suspension was removed with filter paper. Equal volumes of 2% aqueous uranyl acetate (pH 4.0) were added for 20 s to negatively stain the phage particles, and excess solution was removed as described above. Phages were examined by transmission electron microscopy (TEM; LEO 912AB transmission electron microscope; Carl Zeiss) at an 80-kV accelerating voltage, and images were scanned with a Proscan 1,024- by 1,024-pixel charge-coupled device camera at the National Academy of Agricultural Science (Suwon, South Korea). Phages were classified according to the International Committee on Taxonomy of Viruses (ICTV) classification (16).

Sequencing of SPC35 genomic DNA and genome analysis. Extraction of phage DNA was carried out using proteinase K and SDS as previously described (47). Whole-genome sequencing of SPC35 was performed with a Genome Sequencer FLX titanium sequencer (Roche, Mannheim, Germany) and assembled with GS *de novo* assembler software (Roche) at Macrogen Inc., South Korea. Open reading frames (ORFs) of the SPC35 genome were determined using GeneMark.hmm software (37) and the BLAST algorithm (4). The tRNAscan-SE program (36) was used to search for tRNA-coding sequences.

Phage adsorption assay. Exponentially growing cells in early log phase (1 ml, OD_{600} = 1.0 to 1.5) were harvested by centrifugation (13,000 × *g*, 1 min, 4°C), washed with phosphate-buffered saline (pH 7.2), and resuspended in 10 ml LB broth. This bacterial suspension was aliquoted into 10 microtubes (1 ml suspension per 1.75-ml tube) and preincubated at 37°C for 5 min. Then, SPC35 was added (multiplicity of infection [MOI] = 0.01) to each of the 10 tubes with 1-min intervals, and the tubes were incubated until 10 min incubation of the first tube. Cells were immediately removed by centrifugation (13,000 × *g*, 1 min, 4°C) and filtration (0.22-μm-pore-size filter), and the number of unbound free phage particles in each tube was determined by overlay assay. The baseline number was determined by adding the same concentration of SPC35 to fresh LB broth that was not inoculated with host bacteria and incubating the broth at 37°C for 10 min.

Bacterial challenge assay. For the bacterial challenge assay, 50 ml fresh LB broth was inoculated with an overnight culture of *S.* Typhimurium SL1344 or *E. coli* MG1655 (1% inoculum), followed by incubation at 37°C at 220 rpm until the OD_{600} was 0.5. SPC35 stock dilutions (100 μl) were then added (MOI = 0.1, 1, or 10) to these cultures, which were incubated for another 24 h. Bacterial growth was monitored by measuring the OD_{600} at various time points. As a negative control, one bacterial culture was inoculated with 100 μl SM buffer instead of SPC35.

Isolation and subculturing of SPC35-resistant mutants. To isolate SPC35-resistant mutants, the high-titer overlay assay was performed as previously described (14, 30). Briefly, overnight cultures of the host bacteria were serially diluted (10-fold), and 100 μl of each dilution was added to 5 ml soft LB agar (0.4% agar). Then, 100 μl of SPC35 suspension (about 2 × 10^10 PFU/ml) was added (MOI ≥ 10), and these mixtures were poured onto LB agar plates and incubated for 24 h at 37°C for the standard overlay assay.

Five colonies from each resultant plate of *S.* Typhimurium SL1344 and *E. coli* MG1655 were picked with sterilized loops. Each colony was streaked onto a fresh LB agar plate and simultaneously inoculated into 3 ml LB broth, followed by overnight incubation at 37°C. The spotting assay was used to verify the phage resistance. These procedures were repeated at least three times to isolate a single colony and evaluate maintenance of phage resistance.

Amplification and DNA sequencing of *btuB*. The *btuB* genes of *E. coli* MG1655 and *S.* Typhimurium were amplified by PCR using the following primers: for *E. coli* MG1655, primers MG btuB-CF (5′-GCA TGC TCA TCA GAT GTC CAG ATC T-3′) and MG btuB-CR (5′-AAG CTT ACC AGC ACG GTG GGA C-3′), and for *S.* Typhimurium, primers btuB-CF (5′-GCA TGC TCA GTG GAT GTC CAG C-3′) and btuB-CR (5′-AAG CTT ACC AGC ACG GTG GGA C-3′). PCR was carried out using a GeneAmp PCR system 9700 apparatus (Applied Biosystems, Foster City, CA) with the following steps: a predenaturation at 95°C for 7 min, followed by amplification for 30 cycles at 95°C for 45 s, 60°C for 45 s, and 72°C for 140 s and a final extension at 72°C for 7 min. The PCR products were analyzed by 1% agarose gel electrophoresis with 0.5× TAE buffer (20 mM Tris-acetate, 0.5 mM EDTA [pH 8.0]).

The *btuB* genes from SPC35-resistant *E. coli* mutants were sequenced by primer walking using the oligonucleotides SPC35-1F (5′-GGG TGA GTG GTT CTG CC-3′), SPC35-1R (5′-CCG TAG GAA GCA ATG AAG CG-3′), SPC35-2F (5′-CCC TGA ATC TCC AGA CAA CC-3′), and SPC35-2R (5′-GCT CTC TGC TAT TCC GTT ACTC-3′) and primers MG btuB-CF and MG btuB-CR, described above. DNA sequencing was performed with an ABI Prism 3730XL DNA analyzer (Applied Biosystems) at Macrogen Inc., South Korea.

Nucleotide sequence accession number. The GenBank accession number of SPC35 is HQ406778.

RESULTS

Isolation of SPC35 and host range determination. Nine bacteriophages that formed clear plaques on *S.* Typhimurium SL1344 were isolated. The ability of these phages to infect both *S.* Typhimurium SL1344 and *E. coli* MG1655 was confirmed by spotting phage lysate onto bacterial lawns. One phage, SPC35,
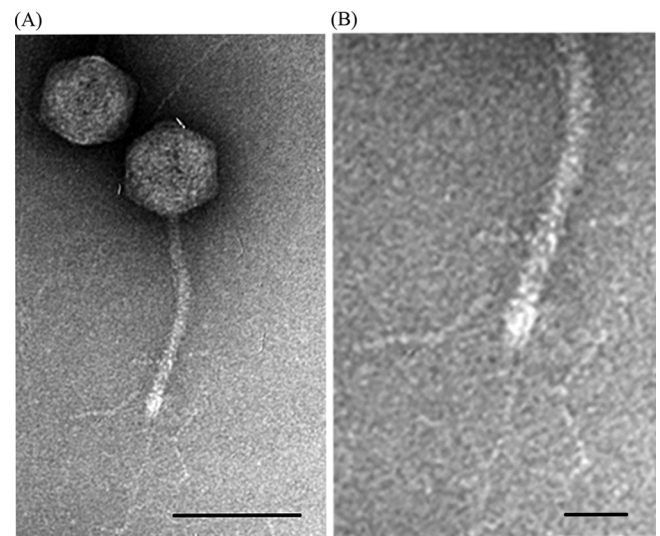

FIG. 1. Transmission electron micrographs of phage SPC35. Bars, 100 nm (A) and 25 nm (B).

formed clear plaques against both bacteria and was selected for further experiments. *E. coli* MG1655 was chosen to propagate SPC35 because plaques produced in this strain were larger and clearer than those produced in *S.* Typhimurium SL1344.

An SPC35 stock (about 2.8 × 10^11 PFU/ml) was diluted 10-fold in SM buffer and analyzed by the spotting assay to determine the host range. All seven *S.* Typhimurium strains, one *Salmonella* Enteritidis isolate, and four *Salmonella enterica* isolates tested were sensitive to SPC35. In addition, SPC35 produced single plaques or bacterial growth inhibition zones with 23 of 40 *E. coli* strains (Table 1).

SPC35 morphology. Morphological analysis by TEM revealed that phage SPC35 has a head with an icosahedral symmetry and a long tail without a contractile sheath, indicating that it belongs to the family *Siphoviridae*. The isometric head of SPC35 had a mean diameter of 70 nm, and the long noncontractile tail was 154 nm by 10 nm (Fig. 1), which closely resembled the morphology of bacteriophage T5 (head diameter, 60 nm; tail, 150 nm by 8 nm).

Sequencing and organization of the SPC35 genome. Only one contig of 108,778 bp (average G+C content, 39.24%) was constructed with 99.97% of bases with a quality score of ≥40. However, the 4,548 nucleotides at the left end of the contig and the 5,025 nucleotides at the right end of the contig were covered by an average read depth of 125.05, while the nucleotides in the other region were covered by an average read depth of 69.18, suggesting that SPC35 contains terminally redundant DNA at both ends of its genome. Accordingly, the SPC35 genome was composed of 118,351 bp with a terminally repeated 9,573 bp and exhibited an average G+C content of 39.39%. One hundred thirty-seven of the 145 predicted putative ORFs were closely related to those of T5 or T5-like phages; thus, phage SPC35 was classified to be a T5 group phage (Fig. 2; see Fig. S1 and Table S1 in the supplemental material). Similar to the genomes of these phages, the SPC35 genome
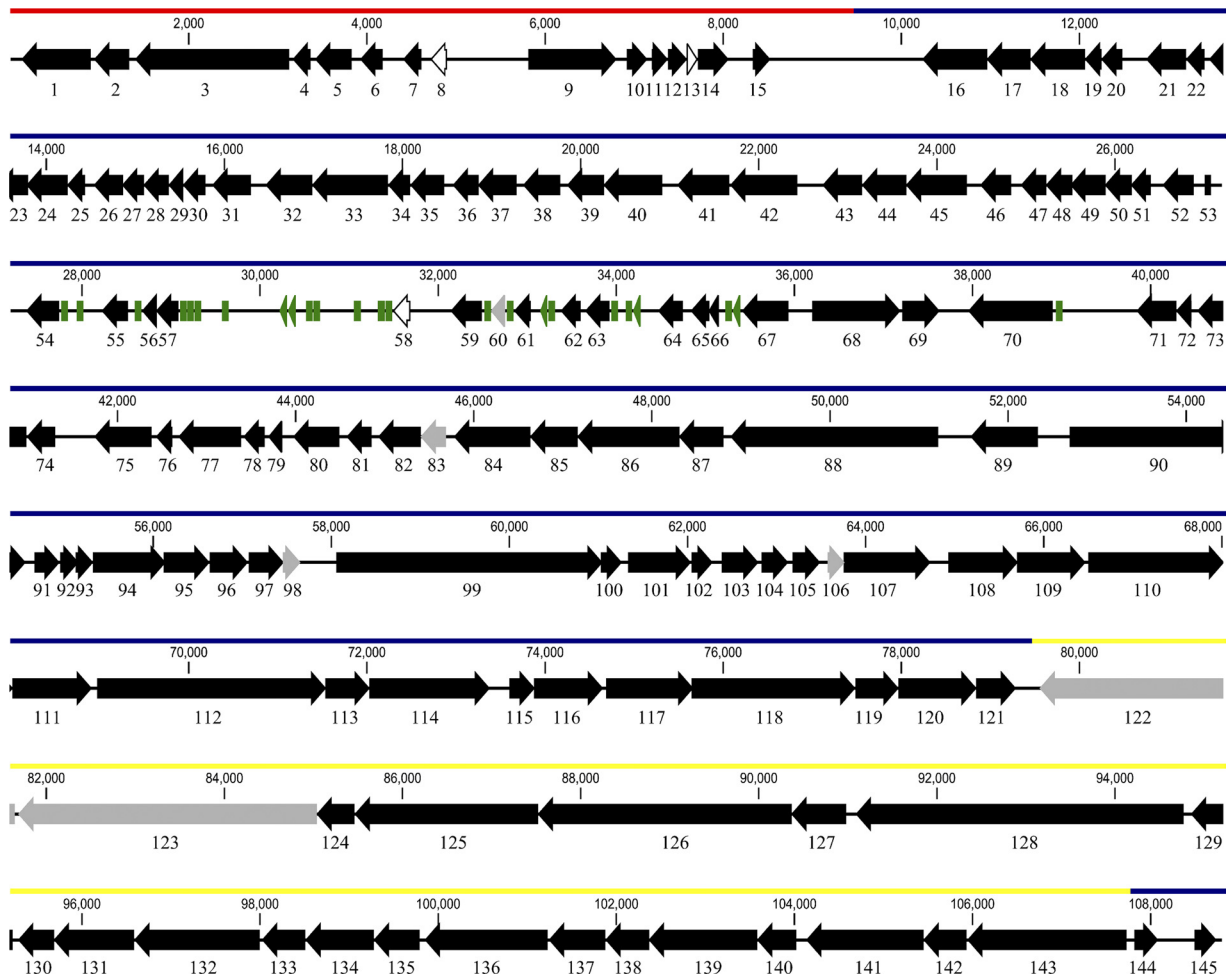
FIG. 2. SPC35 genome map excluding the terminally redundant DNA sequence at the right end. The predicted ORFs with their directions of transcription and tRNA-coding sequences are indicated by colored arrows: black, homologous to T5 group phages (T5, BF23, H8, and EPS7); gray, homologous to other microorganisms; white, no homology; green, tRNA-coding sequences. The red, blue, and yellow lines above the arrows indicate the pre-early, early, and late regions, respectively.

was divided into pre-early, early, and late regions by gene function (Fig. 2).

The pre-early region of the SPC35 genome, which appears to be transferred into the host bacteria in the first-step transfer, contains 15 ORFs that encode various host enzyme inhibitors, including inhibitors for host DNA, RNA, and protein synthesis and gene products that degrade host DNA. ORF10, ORF11, ORF12, ORF14, and ORF15 showed high similarities to ORFs in the pre-early region of T5 and EPS7, which encode these inhibitory proteins (26, 54). In addition, the gene products of ORF1, ORF3, ORF4, and ORF5 were highly homologous to deoxynucleoside 5′-monophosphatase (DMP; 97%), A1 protein (98%), probable A1 protein precursor (98%), and A2 protein (97%) of T5, respectively. These proteins are required for the second-step transfer of the T5 phage genome (54).

The two-step genome transfer mechanism of T5 is facilitated by terminally redundant DNA sequences spanning the pre-early region, a common feature of T5-like phages (26). Similar to T5 or T5-like phages, the terminally repeated 9,573 nucleotides of the SPC35 genome spanned the pre-early region (Fig.

2; see Fig. S1 in the supplemental material) and appeared to facilitate the two-step transfer of the SPC35 genome.

The early region of the SPC35 genome encodes a variety of proteins thought to participate in DNA replication, transcription, recombination, metabolism, signal transduction, and cell lysis; most of these genes are highly homologous to T5 genes. For instance, the putative gene products of ORF110, ORF111, and ORF112 are replicative DNA helicase, DNA replication primase, and DNA polymerase, respectively, with 99% identity to the corresponding T5 genes. ORF39, ORF40, and ORF73 encode phage lysis-related proteins, including lysozyme (endolysin, 80% similarity to the T5 gene), putative holin (97% similarity to the T5 gene), and spore-cortex-lytic enzyme precursor (97% similarity to the T5 gene), respectively. In addition, the repertory of 24 identified tRNA-coding sequences in the early region of the SPC35 genome was identical to that of the T5 genome, except that the tRNA-coding sequence for Val was absent and an additional tRNA-coding sequence for Lys was present in the SPC35 genome. We could not find any genes involved in lysogen formation.
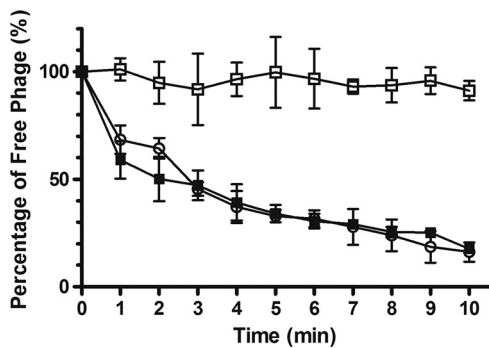
FIG. 3. SPC35 adsorption of *S.* Typhimurium SL1344 (open circles), Δ*btuB* (open squares), and Δ*btuB*(pMS100) (closed squares). Exponentially growing cells were infected with SPC35 (MOI, 0.01) and incubated at 37°C. After centrifugation and filtration, the phage titer in the filtrate was determined by standard overlay assay. The percentage of free phage particles was calculated as follows: (phage titer in the supernatant of SPC35-infected bacterial suspension/phage titer in the supernatant of SPC35-inoculated LB broth) × 100. The results are expressed as means and standard deviations of triplicate assays.

The late region of the SPC35 genome was composed of 24 ORFs. The majority of these ORFs encode structural and morphogenesis proteins, such as head and tail proteins, a portal protein, and a receptor-binding protein. The putative prohead protease, portal protein, terminase (large subunit), and putative SciB protein, encoded by ORF137, ORF139, ORF141, and ORF142, respectively, were nearly identical to the corresponding T5 proteins, whereas tail proteins Pb3 and Pb4, major tail protein, and major head protein precursor, encoded by ORF126, ORF125, ORF132, and ORF136, respec-

tively, showed greater homology to EPS7 proteins. In addition, ORF143, encoding a receptor-binding protein, and ORF144, encoding a receptor-blocking protein, were highly homologous to those of BF23 (84% and 99% identities, respectively), suggesting a common host receptor for BF23 and SPC35.

BtuB acts as a receptor for SPC35. Because the morphological characteristics and genome of SPC35 were similar to those of T5 group phages, we investigated several known receptors of T5-like phages for SPC35 infection. *S.* Typhimurium SL1344 deletion mutants, lacking *fhuA*, *btuB*, or *fepA* (26), were tested for susceptibility to SPC35 by the spotting assay. The only mutant that SPC35 could not infect was the *btuB* deletion mutant, indicating a crucial role for BtuB in the infection process of SPC35. Complementation of the *btuB* mutation by pMS100 harboring the wild-type (WT) *btuB* gene restored the susceptibility of SL1344 to SPC35.

The requirement of BtuB in the initial step of SPC35 infection was further assessed by the phage adsorption assay of WT, mutant, and complemented strains. As shown in Fig. 3, phage adsorption by *S.* Typhimurium SL1344 was completely abolished by the *btuB* mutation. Complementation of the *btuB* mutation with pMS100 restored infection to the levels seen with WT *btuB*, resulting in the gradual reduction of free phage particles. These results strongly suggest that BtuB is the host receptor for phage SPC35.

Differential responses of *S.* Typhimurium and *E. coli* to SPC35 infection. To assess the ability of SPC35 to lyse host bacteria in broth, we evaluated the growth of host bacteria in the presence of SPC35. The effect of SPC35 infection on the growth of *S.* Typhimurium SL1344 differed markedly from that on the growth of *E. coli* MG1655 (Fig. 4). SPC35 caused
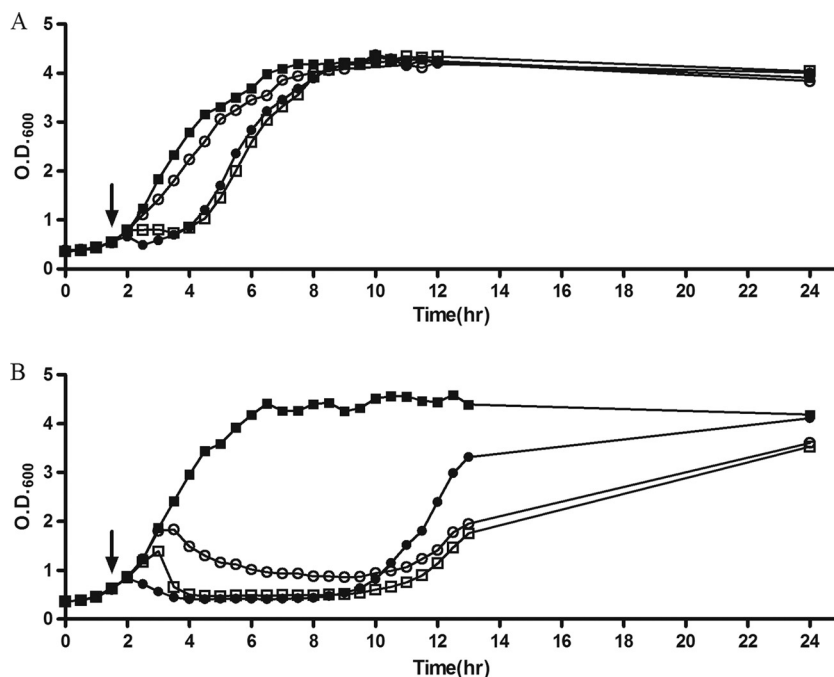

FIG. 4. Bacterial challenge assay with SPC35 to *S.* Typhimurium SL1344 (A) and *E. coli* MG1655 (B). SPC35 (100 μl) was added at an MOI of 0.1 (open circles), an MOI of 1 (open squares), or an MOI of 10 (closed circles) to the bacterial culture after 1.5 h incubation (vertical arrows, OD_{600} = 0.5 to 0.6). A bacterial culture inoculated with SM buffer instead of SPC35 was used as the negative control (closed squares).
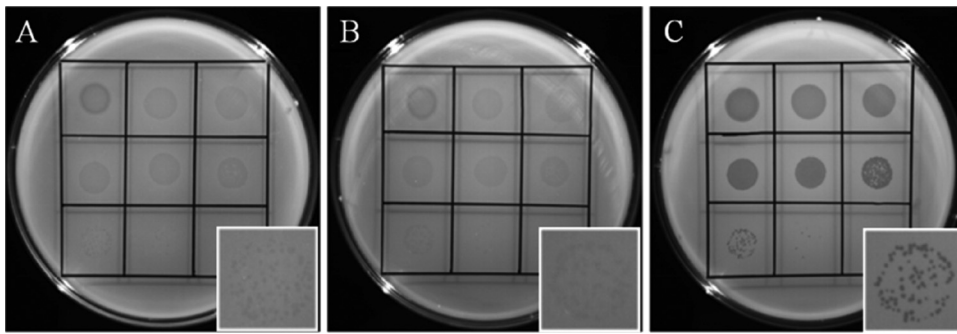
FIG. 5. SPC35 was spotted onto plates of SPC35-resistant *S.* Typhimurium isolates obtained after a single streaking (A), the second streaking (B), and the third streaking (C). Insets at the bottom right of each panel show individual plaques.

growth retardation in *S.* Typhimurium SL1344 rather than growth inhibition. Infection of SL1344 at an MOI of 0.1 produced only slight growth retardation, and an MOI of 1 or 10 produced growth retardation of about 2 h (Fig. 4A). However, in *E. coli* MG1655, infection with SPC35 at an MOI of 0.1 began to suppress host growth significantly about 2 h after infection. Growth inhibition was maintained until approximately 10 h after phage infection, after which bacterial growth eventually resumed (Fig. 4B). Several isolates from this culture obtained after sequential streaking on fresh LB agar plates showed an SPC35 insensitivity in the standard spotting assay (data not shown), indicating the generation of phage-resistant mutants. Addition of SPC35 at an MOI of 1 or 10 also suppressed *E. coli* MG1655 growth, but phage-resistant mutants appeared more rapidly at an MOI of 10.

The differential responses to SPC35 infection by *S.* Typhimurium and *E. coli* were also observed on solid media. In the high-titer (ca. 2×10^{10}-PFU/ml) overlay assay, colonies of various sizes within the overall confluent lysis zone were observed on the *E. coli* MG1655 plate. In contrast, the *S.* Typhimurium SL1344 plate produced a bacterial lawn without any visible lysis zones or plaques (data not shown). However, when 10-fold dilutions of *S.* Typhimurium were incubated with the same concentration of SPC35, several putative phage-resistant colonies were formed at an MOI of 10^6.

SPC35 resistance is heritable in *E. coli* mutants but not in *S.* Typhimurium mutants. Five of the SPC35-resistant *S.* Typhimurium colonies and five of the SPC35-resistant *E. coli* colonies were picked from each of the high-titer overlay plates; these colonies were streaked three times onto fresh LB agar plates in the absence of SPC35 to isolate a single colony. At each streaking step, the newly formed colonies were tested by spotting assay to verify phage resistance. Interestingly, persistence of phage resistance differed between the putative *E. coli* and *S.* Typhimurium mutants. All five putative *E. coli* mutants maintained their resistance throughout the sequential streaking, resulting in no plaques on the spotted lawns. In contrast, all five putative *S.* Typhimurium mutants showed SPC35 sensitivity after the first streaking. However, SPC35 formed relatively turbid plaques in the spotting assay performed with colonies obtained after the first or second streaking but formed clear plaques when the assay was performed with colonies obtained after the third streaking (Fig. 5).

Disruption of the *btuB* gene by IS2 results in heritable resistance to SPC35 in *E. coli*. To determine whether the SPC35-resistant *E. coli* isolates had lost their ability to synthesize functional BtuB protein because of mutation, we analyzed the *btuB* genes of these isolates by PCR amplification. Analysis of the PCR products by agarose gel electrophoresis showed fragments of about 3,500 bp from all mutants except SPC35^RMG-M2 (Fig. 6). The size of the PCR product in WT *E. coli* MG1655 was about 2,200 bp; therefore, these results indicated that the *btuB* gene was mutated by insertion of a 1,300-bp DNA fragment in isolates SPC35^RMG-L1, SPC35^RMG-M1, SPC35^RMG-S1, and SPC35^RMG-S2. DNA sequencing of these amplified fragments revealed insertion sequence (IS) IS2 in the *btuB* genes of all four mutants; however, its orientation and insertion site differed (Table 2 and Fig. 7). IS2 was oriented in the same direction as *btuB* transcription in SPC35^RMG-L1 and SPC35^RMG-M1, but the reverse orientation was observed in SPC35^RMG-S1 and SPC35^RMG-S2. The IS2 insertion in SPC35^RMG-L1 was 901 nucleotides from the *btuB* start codon but 1,687, 1,001, and 939 nucleotides from the start codon in SPC35^RMG-M1, SPC35^RMG-S1, and SPC35^RMG-S2, respectively. In these four mutants, the flanking sequence of IS2 insertion sites had a duplicated sequence of 5 bp, but there is no common noticeable percent G+C content difference in these flanking sequences (Table 2). Complementation of the *btuB*::IS2 mutant clone, SPC35^RMG-L1, with a plasmid containing the intact
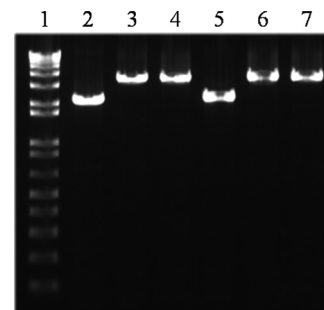

FIG. 6. PCR analysis of the *btuB* gene in SPC35-resistant *E. coli* mutants. Lane 1, DNA ladder; lane 2, *E. coli* MG1655; lane 3, SPC35^RMG-L1; lane 4, SPC35^RMG-M1; lane 5, SPC35^RMG-M2; lane 6, SPC35^RMG-S1; lane 7, SPC35^RMG-S2.

TABLE 2. DNA sequences at insertion site of IS2

Strain or IS2	Flanking sequence[a] (% G+C content)	Left end-IS2-right end		Flanking sequence (% G+C content)	IS2 orientation[b]
E. coli MG1655	ATTACAACTACGATCCCCAT			TATGGTCGTTATGAT	
SPC35^R MG-L1	ATTACAACTACGATCCCCAT (40)	TAGACTGCGCCCCTG	. . . ATAGGGGCAAATCCA	CCCATTATGGTCGTTATGAT (40)	+
SPC35^R MG-M1	ATCAAACCGTTAAAATGGGC (40)	TAGACTGCGCCCCTG	. . . ATAGGGGCAAATCCA	TGGCGGTGTGAGCTTGTGG (65)	+
SPC35^R MG-S1	GTTATGTTGAGGATGGATAT (35)	TGGATTTGCCCCTAT	. . . CAGGGGGCCAGTCTA	GATATGATCAACGTAATACC (35)	−
SPC35^R MG-S2	TCGGCGACGCTCGATGAGAT (60)	TGGATTTGCCCCTAT	. . . CAGGGGGCCAGTCTA	GAGATGAAGCAATACACCGT (45)	−
IS2A[c]	ACGAATAAACGCATAATTAC (30)	TGGATTTGCCCCTG	. . . ATAGGGGCAAATCCA	ATTACCTATCAGGCAGTTTG (40)	+
IS2D	ACATCATGGGACCAAGAACCC (50)	TGGATTTGCCCCTAT	. . . CAGGGGGCCAGTCTA	AACCCCAGAACTTACTTATG (40)	+
IS2E[d]	CACTGGATGACGAACTGGCC (60)	Truncated IS2		CAGGTGAACTACGCTCTCT (55)	+
IS2F	AACAAATCCGCATTCGTGGC (50)	TGGATTTGCCCCTAT	. . . CAGGGGGCCAGTCTA	GTGGCGAAGCATCCTCCCGT (65)	−
IS2H	AGTAAATTCCAATTGTTTAT (20)	TGGATTTGCCCCTAT	. . . CAGGGGGCCAGTCTA	AACTTGCTCTTTTCTCTCGG (40)	−
IS2I	TATTGTTCATTATCCGTTAT (25)	TAGACTGCGCCCCTG	. . . ATAGGGGCAAATCCA	GTTATCATCATTGGTTGTGC (40)	+
IS2K	AGGGATTTGATGGTCCCTTG (50)	TAGACTGCGCCCCTG	. . . ATAGGGGCAAATCCA	CCTTGTGCTGATATGAATAC (40)	+

[a] The 5-bp flanking sequence duplications are underlined. Note that IS2E and IS2H do not have these duplication sequences.
[b] +, IS2 inserted in the same orientation as the direction of btuB transcription; −, IS2 inserted in the opposite orientation as the direction of btuB transcription.
[c] Sequences for IS2 elements have been retrieved from GenBank accession no. U00096.
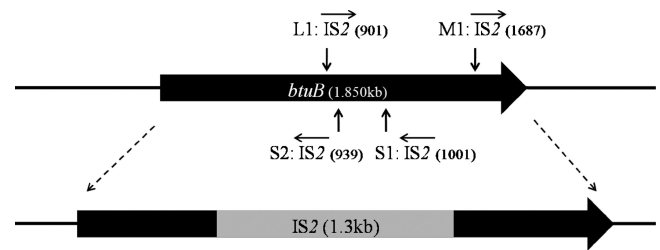[d] IS2E is a truncated element that does not contain an insC (orfA) gene.

FIG. 7. Schematic representation of IS2 insertion sites in the btuB gene of SPC35-resistant E. coli mutants. Vertical arrows indicate insertion of IS2, and the corresponding insertion sites are shown in parentheses. Horizontal arrows indicate the orientation of IS2.

btuB gene resulted in the formation of clear plaques of SPC35 in the spotting assay (data not shown). This result strongly suggested that the IS2 insertion in the btuB gene provided the SPC35 resistance in E. coli. The amplified btuB fragment of SPC35^R MG-M2 was the same size as that of WT E. coli MG1655; however, one nucleotide (G) was deleted among the five G nucleotides from nucleotides 325 to 329 from the start codon.

In contrast, PCR analysis and sequencing of the btuB gene of SPC35-resistant S. Typhimurium isolates using primers btuB-CF and btuB-CR revealed no mutation in the btuB gene.

DISCUSSION

Using S. Typhimurium SL1344 as the host bacterium, we isolated a novel bacteriophage, SPC35, which can infect Salmonella species and E. coli. Morphological analysis by TEM revealed that SPC35 has an icosahedral head and noncontractile tail, indicating that it belongs to the family Siphoviridae (Fig. 1). Sequencing and genome analysis revealed that the SPC35 genome was highly homologous to the genomes of T5 group phages T5, BF23, H8, and EPS7 (26, 45).

Attachment to the host cell is the initial step of phage infection. Various components of the host bacteria, such as flagella, fimbriae, lipopolysaccharide (LPS), and outer membrane proteins, are used as bacteriophage receptors (50). Phage T5 uses the ferrichrome outer membrane transporter FhuA as a receptor (45). Phages BF23 and EPS7 utilize BtuB, the outer membrane protein involved in vitamin B_{12} uptake (26), and H8, which binds to the host ferric enterobactin receptor FepA (45). In the present study, mutant and genome analysis revealed that BtuB is the host receptor for SPC35. Although the SPC35 genome was highly homologous to the T5 genome (see Fig. S1 in the supplemental material), the putative receptor-binding protein of SPC35, which interacts with its corresponding host receptor, was more similar to that of BF23 (amino acid homology for BF23, 84% identity and 91% positive; amino acid homology for T5, 29% identity and 43% positive).

Several strains of bacteria, including S. enterica isolate 3605, E. coli FS575, and E. coli W3110, as well as colonies obtained after streaking of the SPC35-resistant S. Typhimurium strain once or twice in the absence of SPC35, produced turbid plaques, even though the SPC35 genome does not appear to contain genes involved in lysogen formation (see Table S1 in the supplemental material). These results may be the result of

inefficient lysis of the host bacteria or some unknown phage resistance-related factor(s).

The bacterial challenge assay with *S.* Typhimurium and *E. coli* showed that host bacteria could not be completely eliminated at any MOI tested (Fig. 4). This result can be explained by the possibility that a proportion of the initial bacterial population was resistant to the phage or by the rapid appearance of resistant mutants. Carey-Smith et al. (11) and Fischer et al. (17) proposed that the growth retardation in the presence of bacteriophage occurs when only a subpopulation of the host bacteria is susceptible to phage infection, such as the 15% of *E. coli* O157:H7 Mu^L isolates that were resistant to phage PP01 (17). In contrast, Kocharunchitt et al. (32) suggested that incomplete lysis of phage-treated bacteria was due to physiologic and/or genetic modification of host cells during phage infection. In the present study, we found that the physiologic and/or genetic modification of host cells during phage infection differed between genera of host bacteria. In most of the SPC35-resistant *E. coli* isolates, an IS*2* insertion disrupted the *btuB* gene, whereas the SPC35-resistant *S.* Typhimurium isolates had intact *btuB* genes and only transient phage resistance.

Insertion sequences are transposable elements (typically 700 to 2,000 bp) present in many bacterial chromosomes and plasmids (15). IS*2*, which naturally occurs in *E. coli* K-12, is a 1,327-bp insertion sequence (19, 46) of the IS*3* family, which consists of IS*3*, IS*150*, IS*911*, and other elements (38). IS*3* family members contain only two open reading frames, *orfA* and *orfB*, from which the fusion protein transposase, OrfAB, is generated by a −1 translational frameshift; its transposition is mediated by this transposase via circular and linear intermediates (24, 34). Mutations by IS*2* insertion have been reported in both regulatory regions and coding regions of target genes; for example, the promoter regions of the *galOPETK* and *lac* operons and the coding sequences of *galK*, *relA*, and *hemB* have been mutated by IS*2* (35). In the present study, all of the IS*2* elements in SPC35-reistant *E. coli* mutants were inserted in the *btuB*-coding region (Fig. 7). In these mutants, IS*2* was flanked by 5-bp sequence duplications, which normally form during IS*2* integration into its target gene (19); IS*2* showed no apparent preference for percent G+C content in target sequences (Table 2).

Only a few studies have reported phage-resistant mutants with IS insertions in their receptor genes. The *tonB* gene of *E. coli*, which encodes a protein that assists in phage T1 infection as well as the colicin B reaction, was spontaneously inactivated by IS*1*, IS*2*, and IS*10* in the presence of colicin B (31). *Vibrio cholerae* phage K139 uses the O1 antigen of LPS as the host receptor for infection. K139-resistant *V. cholerae* mutants were reported to have IS*1004* inserted into the O-antigen biosynthesis gene cluster (*manB*, involved in the biosynthesis of perosamine, and *wbeW*, which encodes a glycosyltransferase) (42).

Salmonella Typhimurium LT2 contains only one type of IS element, IS*200*, whereas *E. coli* K-12 contains 8 to 12 different IS elements, including IS*2*. Moreover, IS*200* transposes less frequently than *E. coli* K-12 IS elements (15). Thus, nonheritable phase-variable physiologic changes stimulated by phage infection may be a more common source of resistance than IS*200* insertion in *S.* Typhimurium.

IS elements are widespread; therefore, mutants spontaneously resistant to phage due to IS element insertion into genes required for phage infection are a problem. However, the low transposition frequency of IS elements suggests that this problem could be overcome with phage cocktails consisting of various virulent phages that use different host receptors. Simultaneous insertion of IS elements into every gene encoding a host receptor targeted by the phage cocktail is unlikely. Thus, identification of the phage receptor is useful for the production of better phage cocktails.

The transience of SPC35 resistance in *S.* Typhimurium strains was not due to pseudolysogeny, as described by Ackermann and DuBow (1), because we did not observe spontaneous production of phages from the SPC35-resistant *S.* Typhimurium isolates. PCR analysis also revealed that these isolates did not contain the SPC35 genome (data not shown). Differential resistance is not specific to SPC35; *E. coli* and *Salmonella* also showed differential resistance to several other phages isolated in our lab (data not shown). Transient phage resistance similar to that observed in *S.* Typhimurium SL1344 was also reported in *E. coli* O157:H7 and other *Salmonella* spp. (32, 49), but the underlying mechanisms for this phenomenon are not known. The mechanism is under investigation in our laboratory.

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