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Genomic Investigation of Lysogen Formation and Host Lysis Systems of the *Salmonella* Temperate Bacteriophage SPN9CC

Hakdong Shin,[a] Ju-Hoon Lee,[b] Hyunjin Yoon,[c] Dong-Hyun Kang,[a] Sangryeol Ryu[a]

Department of Food and Animal Biotechnology, Department of Agricultural Biotechnology, Center for Food and Bioconvergence, and Research Institute for Agriculture and Life Sciences, Seoul National University, Seoul, South Korea[a]; Department of Food Science and Biotechnology, Graduate School of Biotechnology, Kyung Hee University, Yongin, South Korea[b]; Department of Food Technology and Services, Eulji University, Seongnam, South Korea[c]

To understand phage infection and host cell lysis mechanisms in pathogenic *Salmonella*, a novel *Salmonella enterica* serovar Typhimurium-targeting bacteriophage, SPN9CC, belonging to the *Podoviridae* family was isolated and characterized. The phage infects *S.* Typhimurium via the O antigen of lipopolysaccharide (LPS) and forms clear plaques with cloudy centers due to lysogen formation. Phylogenetic analysis of phage major capsid proteins revealed that this phage is a member of the lysogen-forming P22-like phage group. However, comparative genomic analysis of SPN9CC with P22-like phages indicated that their lysogeny control regions and host cell lysis gene clusters show very low levels of identity, suggesting that lysogen formation and host cell lysis mechanisms may be diverse among phages in this group. Analysis of the expression of SPN9CC host cell lysis genes encoding holin, endolysin, and Rz/Rz1-like proteins individually or in combinations in *S.* Typhimurium and *Escherichia coli* hosts revealed that collaboration of these lysis proteins is important for the lysis of both hosts and that holin is a key protein. To further investigate the role of the lysogeny control region in phage SPN9CC, a Δ*cI* mutant (SPN9CCM) of phage SPN9CC was constructed. The mutant does not produce a cloudy center in the plaques, suggesting that this mutant phage is virulent and no longer temperate. Subsequent comparative one-step growth analysis and challenge assays revealed that SPN9CCM has shorter eclipse/latency periods and a larger burst size, as well as higher host cell lysis activity, than SPN9CC. The present work indicates the possibility of engineering temperate phages as promising biocontrol agents similar to virulent phages.

Food poisoning is generally caused by the intake of a food or drink contaminated with food-borne pathogenic bacteria, such as *Salmonella*, *Escherichia coli*, *Listeria*, and *Campylobacter* (1). *Salmonella* causes salmonellosis with various symptoms, such as diarrhea, vomiting, high fever, and even death (2, 3). In the United States, more than 1.4 million cases of salmonellosis have been reported every year, and the number has increased by more than 10% annually in recent years (1, 3, 4). Although antibiotics have been widely used to control the pathogen responsible for salmonellosis, multidrug-resistant *Salmonella* strains, such as *Salmonella enterica* serovar Typhimurium DT104, have appeared (5, 6).

Because of the emergence of antibiotic-resistant *Salmonella* strains, an approach using bacteriophage has been proposed to control them (7, 8). To take advantage of phage treatment against salmonellosis, it is necessary to characterize *Salmonella* phages phenotypically and genotypically. Moreover, understanding of mechanisms of *Salmonella* host cell infection by *Salmonella*-targeting phages is important for this purpose. The major processes of host infection by phages include phage attachment via a host receptor, control of the host lytic-lysogenic cycle, and the host cell lysis mechanism.

Several *Salmonella* host receptors for phage infection have been experimentally determined and characterized, such as flagella (9, 10), Vi capsular antigen (11), lipopolysaccharide (LPS) (12), and host outer membrane proteins (OmpC [13], BtuB [14, 15], TolC [16], and FhuA [17]). These receptors play a role in the determination of phage host specificity, suggesting that host receptor study would be able to provide novel insights into the mechanisms of phage infection of *Salmonella* host cells. Lambdoid lysogenic phages generally contain a lysogeny control region consisting of *cro*, *cI*, *cII*, *cIII*, *N*, and *Q* (18, 19). Constitutive bacteriophage promoters, P_L and P_R, express N and Cro proteins. N protein binds to all terminators for antitermination. During this early gene expression, CII, CIII, and Q proteins are produced. Among these proteins, the CII-CIII complex activates P_{RE} and P_I promoters, resulting in the lysogenic cycle by the production of integrase and CI protein, which are related to phage genome integration and blocking of all phage gene expression. At this point, if the host HflA proteolytic enzyme is activated in the presence of a low concentration of cyclic AMP because of a sufficient supply of glucose to the host, it digests CII protein such that the CII-CIII complex cannot produce CI protein, resulting in prevention of the lysogenic cycle. Furthermore, Q protein activates gene expression related to phage structure and host cell lysis. Therefore, the study of the lysogeny control region is important to understand the phage lytic/lysogenic cycles in the host. Holin and endolysin are known to be important for host cell lysis (20). Holin creates holes in the cytoplasmic membrane. These holes are used as transport channels for endolysin, which digests the peptidoglycan layer. In addition, Rz/Rz1-like proteins often enhance endolysin activity as endolysin accessory proteins (21).

Salmonella-targeting phage P22 belongs to the family of *Podoviridae* morphologically and has been well characterized to de-

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Address correspondence to Sangryeol Ryu, sangryu@snu.ac.kr.
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velop genetic transfer tools via lysogenization (18, 19). Host receptor studies have revealed that the phage tailspike protein plays a role in the interaction with the host by interacting with the O antigen of LPS in *S.* Typhimurium (22, 23). Complete analysis of the phage P22 genome sequence also revealed the presence of functional genes related to lysogenization and host specificity determination (18, 24). In addition, comparative genomic analysis of P22 and closely related phages revealed the presence of the P22-like phage group (25). This group includes ε34, ST104, ST64T, SE1, c341, and HK620. They share phage morphogenesis and assembly genes for similar morphology and generally infect *Salmonella*, *E. coli*, and *Shigella* in the *Enterobacteriaceae* family. However, while *ant* moron regions in phage P22 have been known to be involved in the regulation of gene expression, these regions are completely or partially missing from other P22-like phages (26). Although the role of this region is not clearly understood, it may be related to lysogeny conversion (27). Further studies of the genomes of these P22-like phages indicate that morphogenesis-related genes are highly conserved, but other genes are variable, suggesting that even though they have similar phage morphologies, the host specificity of these P22-like phages may differ among them. Therefore, further study of these P22-like phages would provide new information about host infection by phages in this group.

To understand the infection mechanisms of the bacteriophage at the genomic level, the complete genome of SPN9CC was analyzed and compared with P22-like phage genomes. In addition, a Δ*cI* mutant of the lysogen-forming P22-like phage SPN9CC was constructed and characterized. This study will be useful for increasing our knowledge of the host infection and lysis mechanisms of P22-like phages, including SPN9CC.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study and gene knockout mutant strains for the host receptor study are listed in Table 1. Prophage-free *Salmonella enterica* serovar Typhimurium LT2C was used for the isolation and propagation of *S.* Typhimurium-targeting phages (28) (Cancer Research Center, Columbia, MO). All of the bacteria listed in Table 1 were cultivated at 37°C for 12 h in Luria-Bertani (LB) broth medium (Difco, Detroit, MI), and the agar medium was prepared by 1.5% agar supplementation (Difco) of the broth medium.

Bacteriophage isolation and propagation. Commercially processed broiler chicken skin samples were collected from the Moran traditional market, Seongnam, South Korea, and used for isolation of *S.* Typhimurium-targeting bacteriophage SPN9CC with *S.* Typhimurium strain LT2C. The basic procedures for the isolation and propagation of bacteriophage SPN9CC were previously described by Shin et al. (29).

Lysogen induction. Selected SPN9CC lysogens of *S.* Typhimurium LT2C were cultivated at 37°C until the optical density at 600 nm (OD_{600}) reached 1.0, and 0.5 μg/ml of mitomycin C (Sigma, St. Louis, MO) was added to the cultures. Then, these cultures were additionally incubated at 37°C for 2 h. After incubation, the cells were removed by centrifugation and filtration, and the supernatant was collected. The spotting assay of this supernatant with *S.* Typhimurium LT2C was conducted to confirm the presence of induced phage SPN9CC.

Electron microscopy. A transmission electron microscope (TEM) was used for morphological analysis of purified phage SPN9CC. This TEM analysis was performed as described by Shin et al. (29). The morphological classification of phage SPN9CC was conducted according to the guidelines of the International Committee on Taxonomy of Viruses (30).

TABLE 1 Host range of SPN9CC bacteriophage

Bacterial host	SPN9CC[a]	Source[b] or reference
Salmonella enterica serovar Typhimurium		
LT2	+++	73
LT2C	+++	28
SL1344	+++	NCTC
UK1	+++	74
ATCC 14028s	+++	ATCC
DT104	+	6
ATCC 43174	++	ATCC
Salmonella enterica serovar Enteritidis ATCC 13076	+	ATCC
Salmonella enterica serovar Paratyphi		
A IB 211	++	IVI
B IB 231	−	IVI
C IB 216	−	IVI
Salmonella enterica Dublin IB 2973	+	IVI
Escherichia coli		
K-12 MG1655	−	75
DH5α	−	ATCC
O157:H7 ATCC 35150	−	ATCC
O157:H7 ATCC 43890	−	ATCC
Gram-negative bacteria		
Shigella flexneri 2a strain 2457T	−	IVI
Shigella boydii IB 2474	−	IVI
Vibrio fischeri ATCC 700601	−	ATCC
Pseudomonas aeruginosa ATCC 27853	−	ATCC
Cronobacter sakazakii ATCC 29544	−	ATCC
Gram-positive bacteria		
Enterococcus faecalis ATCC 29212	−	ATCC
Staphylococcus aureus ATCC 29213	−	ATCC
Bacillus cereus ATCC 14579	−	ATCC
Listeria monocytogenes ATCC 19114	−	ATCC
Salmonella Typhimurium SL1344 mutants		
Δ*flgK* mutant	+++	29
Δ*btuB* mutant	+++	15
Δ*rfaL* mutant	−	31
Δ*rfaL* (*pUHE21-lacIq*::*rfaL*) mutant	+++	31

[a] +++, EOP of 1 to 0.01; ++, EOP of 0.01 to 0.0001; +, EOP of, <0.0001; −, not susceptible to SPN9CC.
[b] NCTC, National Collection of Type Cultures; ATCC, American Type Culture Collection; KCTC, Korean Collection for Type Cultures; IVI, International Vaccine Institute.

Host range determination by spotting assay. The host range and comparative efficiency of plating (EOP) of phage SPN9CC were determined with a spotting assay using *S.* Typhimurium, *S.* Paratyphi, *E. coli*, and other Gram-negative and Gram-positive bacterial strains by the procedure previously described by Park et al. (31).

Genome sequencing and bioinformatic analysis. Genomic DNA of phage SPN9CC was isolated and purified as described by Sambrook and Russell (32). The construction of a genomic DNA library and pyrosequencing with Genome Sequencer FLX (GS-FLX) Titanium (Roche, Mannheim, Germany) were conducted by Macrogen, Seoul, South Korea. The prediction of open reading frames (ORFs) was conducted with Glimmer 3.02 (33), GeneMarkS (34), and FgenesV (Softberry, Inc., Mount Kisco, NY). The prediction of ribosomal binding sites of ORFs was performed with RBSfinder (J. Craig Venter Institute, Rockville, MD). The

TABLE 2 Primers used in this study

Primer	Sequence (5′ to 3′)[a]	Reference
SPN9CC_0042_F	TAAAAGAATTCAAATCCCCTCAATAAAGGGGGTAGAG	This study
SPN9CC_0042_R	TTTTTGTCGACTTATCGCCGCTATTACGCTATTTC	This study
SPN9CC_0043_F	AAAAAGAATTCAAACGCAAAGAGCGTGAGGACAG	This study
SPN9CC_0043_R	TTTTTGTCGACATAATCGCGGTTACTCTGCTCATTG	This study
SPN9CC_0044_F	AAATTGAATTCTTGAGCGTGAAGTCTGTTTGTGGG	This study
SPN9CC_0044_R	AAAAAGTCGACTATGTGATGGAAATTATTTCAGGCATTG	This study
9CC-BRED_C	TCTTAAAAGTGAACTCATCACCACATAACCTTGCAATGCAAAAAGCTTCGC TATGTCATACCAGTTCATTTTCATCCTTAAATTATACA	This study
9CC-BRED_CF	TTGTAGGAATACTTGTCCGCTGTCTTTGATGAGCTTCTTAAAAGTGAACT CATCATGTAGGCTGGAGCTGCTTCG	This study
9CC-BRED_CR	TTTACGATTTGTGACTGTTCTTGTTTGATACAAATTGTATAATTTAAGGAT GAAAATTCCGGGGATCCGTCGACC	This study
9CC-BRED_conf_F	TATCTCATCAGGCCATTGGCTGGCTACAAC	This study
9CC-BRED_conf_R	TAATGACAAACTGCACCACGCGTACAACCG	This study

[a] Specific restriction enzymes used for cloning are underlined. Forward and reverse primers contain EcoRI and SalI sites, respectively.

annotation of predicted ORFs was conducted with BLASTP (35) and InterProScan (36) by using conserved protein domain databases. The GenBank data file was generated with the GAMOLA (37) and Sequin programs (National Center for Biotechnology Information, Bethesda, MD). The phylogenetic analysis of major capsid proteins (MCPs) from bacteriophages, including SPN9CC, was performed with MEGA5 by the neighbor-joining method by using p distance values (38). The program Mobyle was used for comparative codon usage analysis of the *S.* Typhimurium SL1344 host and phage SPN9CC (39). Comparative genomic analysis of SPN9CC with other P22-like phages and visualization were conducted with BLASTN (35) and ACT12 (40).

Expression of the host cell lysis gene cluster. The SPN9CC_0042, SPN9CC_0043, SPN9CC_0044, and SPN9CC_0044_1 genes, encoding holin, endolysin, and Rz/Rz1 endopeptidases, respectively, in the host cell lysis gene cluster were amplified by PCR with the primers listed in Table 2. These PCR products were doubly digested with EcoRI and SalI and cloned into the multiple cloning site of pBAD18 (41) individually or in combination with more than two genes. These cloned plasmids are listed in Table 3. *S.* Typhimurium SL1344 and *E. coli* MG1655 were used as gene expression hosts of the cloned pBAD18 plasmids after transformation. The expression of the cloned genes was induced by the addition of 0.2% (final concentration) arabinose after 2 h of incubation of the subinoculated cultures. To test the lysis activity of host cell lysis proteins during incubation and the induction of the cultures, the OD_{600} was measured every hour.

TABLE 3 Plasmids used in this study

Plasmid	Description	Reference
pBAD18	P_{araC} ColE1 *ori* Amp^r	41
pBAD18-42	pBAD18 expressing SPN9CC_0042	This study
pBAD18-43	pBAD18 expressing SPN9CC_0043	This study
pBAD18-44	pBAD18 expressing SPN9CC_0044	This study
pBAD18-42/43	pBAD18 expressing SPN9CC_0042 and SPN9CC_0043	This study
pBAD18-43/44	pBAD18 expressing SPN9CC_0043 and SPN9CC_0044	This study
pBAD18-42/43/44	pBAD18 expressing SPN9CC_0042, SPN9CC_0043, and SPN9CC_0044	This study
pUHE21-2 $lacI^q$	pMB1 *ori* Amp^r $lacI^q$	76
pUHE21-2 $lacI^q$::*rfaL*	pUHE21-2 $lacI^q$ expressing *rfaL*	31
pUHE21-2 $lacI^q$::*flgK*	pUHE21-2 $lacI^q$ expressing *flgK*	29
pACYC184	p15A *ori* Cm^r Tet^r	77
pMS100	pACYC184 expressing *btuB*	15

Deletion of the *cI* gene from the SPN9CC genome by BRED. The *cI* gene of SPN9CC was specifically deleted by the bacteriophage recombineering of electroporated DNA (BRED) method previously described by Marinelli et al. (42). To delete the *cI* gene by the BRED method, a 200-bp double-stranded DNA substrate containing a 100-bp region upstream and the other 100-bp region downstream of the *cI* target gene was PCR amplified with primers 9CC-BRED_C, 9CC-BRED_CF, and 9CC-BRED_CR (Table 2). An *S.* Typhimurium SL1344 electroporation host with pKD46 encoding recombinase was induced with arabinose and used for electrocompetent cell preparation (43). The phage genomic DNA and 200-bp DNA substrate were coelectroporated into the arabinose-induced electrocompetent cells for homologous recombination. After a 1-h shaking incubation of the transformants at 37°C, 6 ml of 0.4% molten LB top agar containing 200 μl of the transformant culture was overlaid on the 1.5% LB base agar and incubated overnight. Plaques were randomly picked, and plaque PCR was performed with specific primers 9CC-BRED_conf_F and 9CC-BRED_conf_R (Table 2). The plaque PCR products were partially sequenced with the same primers to confirm the deletion of the *cI* gene. Phage SPN9CCM with the *cI* gene deleted was purified by the single-picking method and propagated as described above.

One-step growth curve and bacterial challenge test. *S.* Typhimurium SL1344 was used as the host strain for one-step growth curve determination and a bacterial challenge test. The overall procedures used for the one-step growth curve assay and the challenge test were previously described by Park et al. (31).

Nucleotide sequence accession number. The GenBank accession number of the complete genome sequence and annotation information of bacteriophage SPN9CC is JF900176.1.

RESULTS

Isolation and morphology of phage SPN9CC. For the development of new biocontrol agents, *Salmonella*-targeting bacteriophages were isolated from a commercially processed broiler chicken skin sample with the host strain *S.* Typhimurium LT2C. Of these phages, SPN9CC produced distinct clear plaques with cloudy centers (see Fig. S1A in the supplemental material), suggesting the possibility of lysogen formation in the cloudy center. Mitomycin C treatment of the colonies isolated from the cloudy centers of the clear plaques revealed the induction of phage SPN9CC, confirming lysogen formation (data not shown). TEM morphological observation revealed that this phage has the short tail typical of members of the *Podoviridae* family (see Fig. S1B).

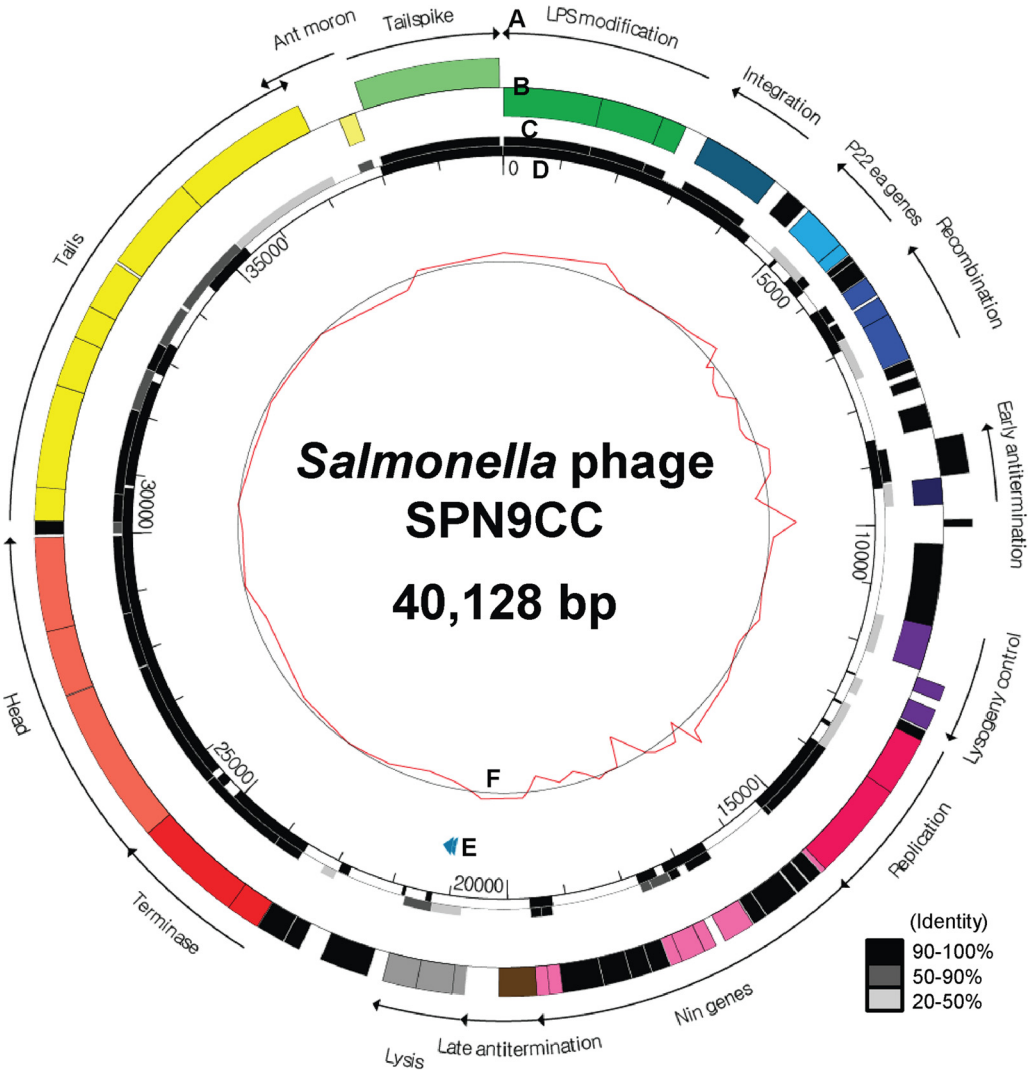

FIG 1 Genome map of phage SPN9CC. (A) Functions of gene clusters. (B) Predicted ORFs by strand. The colors indicate the functions of the gene clusters. Black ORFs encode hypothetical proteins. (C) Comparative analysis of phage SPN9CC and P22 ORFs at the amino acid sequence level. Different degrees of homology between phage SPN9CC and P22 ORFs are indicated by different levels of darkness, as shown in the lower right corner. (D) Comparative genomic analysis of phages SPN9CC and P22 at the DNA sequence level. (E) tRNA prediction is indicated by the blue arrowheads. (F) GC content of phage SPN9CC. The scale values are in base pairs.

Host range and host receptor study. The host range test of phage SPN9CC demonstrated specific inhibition of *S.* Typhimurium, *S.* Paratyphi, and *S.* Dublin. However, various Gram-positive and Gram-negative bacteria, including other *Salmonella* strains, were not inhibited by this phage, suggesting that it specifically infects certain *Salmonella* strains (Table 1). To determine the host receptor for phage SPN9CC, previously constructed mutants of *S.* Typhimurium SL1344 were used, including a Δ*flgK* mutant (*flgK* encodes a flagellar hook-associated protein), a Δ*btuB* mutant (*btuB* encodes a vitamin B_{12} uptake protein), and a Δ*rfaL* mutant (*rfaL* encodes O-antigen ligase) (15, 29, 31). Only the Δ*rfaL* mutant displayed resistance to phage SPN9CC, suggesting that the O antigen of LPS is a host receptor for phage infection. Subsequent complementation of this mutant with the pUHE21-*lacI*^{q}::*rfaL* expression vector (31) confirmed O antigen as a receptor of SPN9CC (Table 1).

Bacteriophage genome analysis. Sequencing of the complete genome of SPN9CC was performed with approximately 90 times coverage by next-generation sequencing (NGS) technology with a 454 pyrosequencer, revealing 40,128 bp with a GC content of 47.33%, 63 putative ORFs, and two tRNAs (tRNA_Thr and tRNA_Asn) (Fig. 1). Comparative analysis of the codon usage preferences of tRNA_Thr of the *S.* Typhimurium SL1344 host and phage SPN9CC indicated a different preference in threonine, suggesting that this tRNA may play a role in the translation of phage mRNA and not of host mRNA. In addition, the gene density was observed to be 1.545 genes/kb and the coding percentage was 90.9%. The average length of each ORF was determined to be 588 bp. A comparative phylogenetic analysis using MCPs from various phages revealed that SPN9CC is closely related to *Salmonella*-targeting P22-like phages such as P22, ST64T, ST104, and ε34 (Fig. 2).

Annotation and functional analysis of the 63 ORFs in this genome revealed that 44 of them have putative functions. Func-

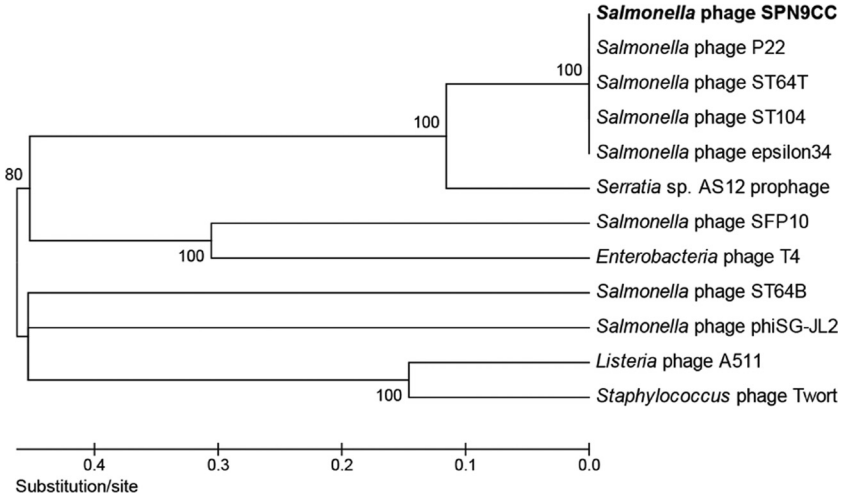

FIG 2 Comparative phylogenetic analysis of MCPs from various bacteriophages. The MCPs were compared with the ClustalW multiple-alignment algorithm, and the phylogenetic tree was generated with MEGA5 by the neighbor-joining method by using p distance values.

tional categorization of these genes revealed 14 groups, such as LPS modification and superinfection exclusion (O-antigen conversion proteins GtrABC and superinfection exclusion protein B), integration (phage integrase), P22 *ea* genes (Eaa and Eai), recombination (Erf recombination protein, Abc1, and Abc2 anti-RecBCD proteins), antitermination (antitermination proteins N and Q), lysogeny control (Cro, CI, and CII), replication (DNA replication protein and helicase), *nin* genes (NinABEFHXZ), host cell lysis (holin, endolysin, and Rz/Rz1 endopeptidases), DNA packaging (terminase large and small subunits), head (portal protein, scaffolding protein, and MCP), tail (DNA stabilization proteins/tail accessory proteins [Gp4, Gp10, and Gp26], head assembly protein, and DNA transfer proteins/ejection proteins), Ant moron (Mnt regulatory protein), and host specificity (tailspike protein).

Comparative genomic analysis of SPN9CC with P22-like phages. Comparative genomic analysis of phage SPN9CC with P22-like phages such as P22 and ε34 revealed that DNA packaging and morphogenesis (heads and tails) gene clusters are highly conserved, indicating that P22-like phages commonly share phage structure genes and belong to the *Podoviridae* family (Fig. 3A). A recent comparative genomic study of P22-like phages supports our analysis result (25). However, the tailspike protein of ε34 differs enough from those of phages P22 and SPN9CC that it most likely has a different host specificity (Fig. 3A). While host range analyses of phages P22 and SPN9CC displayed the same inhibition spectrum (data not shown), the specific infection of *S.* Anatum by phage ε34 substantiates this (44). The lysogeny control region (Cro, CI, and CII) of phage SPN9CC differs from that of phage P22 but is similar to that of phage ε34, suggesting that SPN9CC and ε34 may share lytic/lysogenic decision and lysogen formation mechanisms (Fig. 3B). Comparative analysis of the host cell lysis gene clusters of phages SPN9CC, P22, and ε34s revealed that they are not conserved among them, suggesting that they most likely lyse their host strains in different manners (Fig. 3C). Although the functions of the genes in this gene cluster of phage P22 were experimentally confirmed (45–47), the function of each gene in the host cell lysis gene cluster of phage SPN9CC cannot be deduced from those in the gene cluster of phage P22 because of the low levels of identity between the amino acid sequences encoded by these genes of P22 and phage SPN9CC. To understand the host cell lysis mechanism of phage SPN9CC, the function of each gene in the host cell lysis gene cluster of phage SPN9CC should be confirmed experimentally. Interestingly, the genes in this gene cluster of phage SPN9CC are similar to those of ST104 and even *E. coli* K-12 prophage DLP12, suggesting that they may use the same mechanism for host cell lysis (Fig. 3D). Successful *S.* Typhi cell lysis results obtained with endolysin from *E. coli* phage DLP12 support this (48). However, whereas the amino acid sequence identity levels of host cell lysis proteins, such as holin, endolysin, and Rz/Rz1-like proteins, between two host cell lysis gene clusters in phages SPN9CC and ST104 are extremely high, the functions of the genes in the gene cluster of ST104 have not been experimentally confirmed. Therefore, the expression of these genes in *S.* Typhimurium and *E. coli* host strains needs to be examined to elucidate the functions of all of the genes in the host cell lysis gene cluster of phage SPN9CC and their cooperation effect on host cell lysis.

Function of host cell lysis gene cluster. Interestingly, the high level of amino acid sequence identity of the host cell lysis proteins (except for holin) encoded by the host cell lysis gene clusters of *S.* Typhimurium-targeting phage SPN9CC and *E. coli* K-12 prophage DLP12 suggests that host cell lysis proteins encoded by the genes in this cluster of phage SPN9CC should function in both *Salmonella* and *E. coli*. To elucidate the host cell lysis mechanism of this phage, each gene in this cluster was cloned into pBAD18 and transformed into *S.* Typhimurium and *E. coli* host cells, respectively.

The expression of a single gene encoding holin (SPN9CC_0042) in *S.* Typhimurium resulted in host cell lysis (Fig. 4A). However, the expression of a single gene encoding endolysin (SPN9CC_0043) or Rz/Rz1-like proteins (SPN9CC_0044) in *S.* Typhimurium did not, suggesting that the endolysin needs holin to cross the cytoplasmic membrane. To elucidate their cooperation effects on *S.* Typhimurium host cell lysis, various combinations for the expression of more than two genes were prepared and those genes were coexpressed in *S.* Typhimurium. The expression of combinations of the genes for holin and endolysin or all

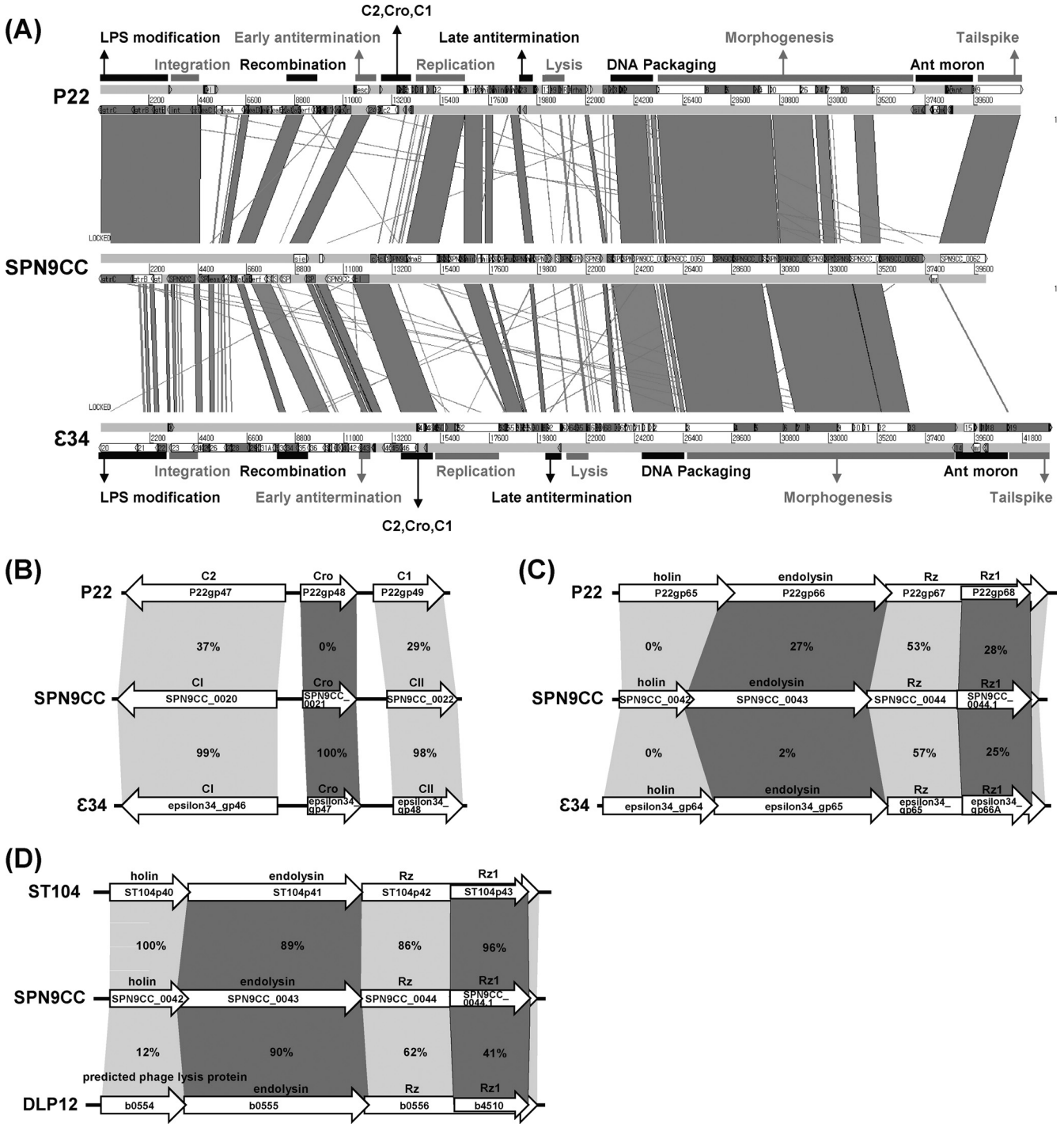

FIG 3 Comparative genomic analysis of P22-like phages (SPN9CC, P22, ST104, and ε34) and *E. coli* K-12 prophage DLP12. (A) Comparative analysis of the complete genome sequences of SPN9CC, P22, and ε34 with BLASTN and ACT12. Black and gray bars indicate the functions of gene clusters in the genomes. (B and C) Comparative analyses of lysogeny control regions (B) and host cell lysis gene clusters (C) in SPN9CC, P22, and ε34. (D) Comparative analysis of host cell lysis gene clusters in SPN9CC, ST104, and *E. coli* K-12 prophage DLP12. The percentages of amino acid identity between homologous genes are indicated.

four cell lysis proteins (holin plus endolysin or holin plus endolysin plus Rz/Rz1-like proteins) in *S.* Typhimurium resulted in much higher host cell lysis efficiency than expression of the holin gene alone (Fig. 4A). However, gene expression combinations without holin (endolysin plus Rz/Rz1-like proteins) did not lyse the host cells, suggesting that holin is a key protein for the lysis of *S.* Typhimurium (Fig. 4A).

However, the expression of these genes in *E. coli* host cells displayed different host cell lysis patterns (Fig. 4B). As for the *S.* Typhimurium host cells, endolysin alone did not contribute to the

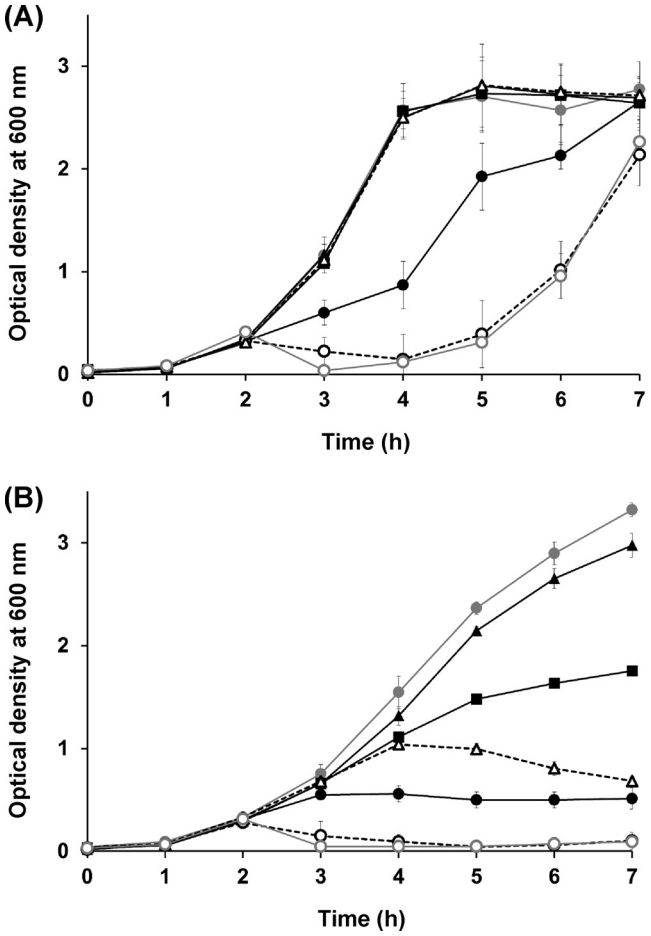

FIG 4 Confirmation of the host cell lysis system of phage SPN9CC via the expression of host cell lysis genes encoding holin, endolysin, and Rz/Rz1 endopeptidases in *S.* Typhimurium SL1344 (A) and *E. coli* MG1655 (B). Closed gray circles represent the negative control without gene expression. Closed black circles, triangles, and squares indicate the expression levels of the SPN9CC_0042 (holin), SPN9CC_0043 (endolysin), and SPN9CC_0044/0044.1 (Rz/Rz1) genes, respectively. Open black circles and triangles with dotted lines indicate the coexpression of SPN9CC_0042/0043 and SPN9CC_0043/0044/0044.1, respectively. Open gray circles indicate the coexpression of all of the genes, SPN9CC_0042/0043/0044/0044.1.

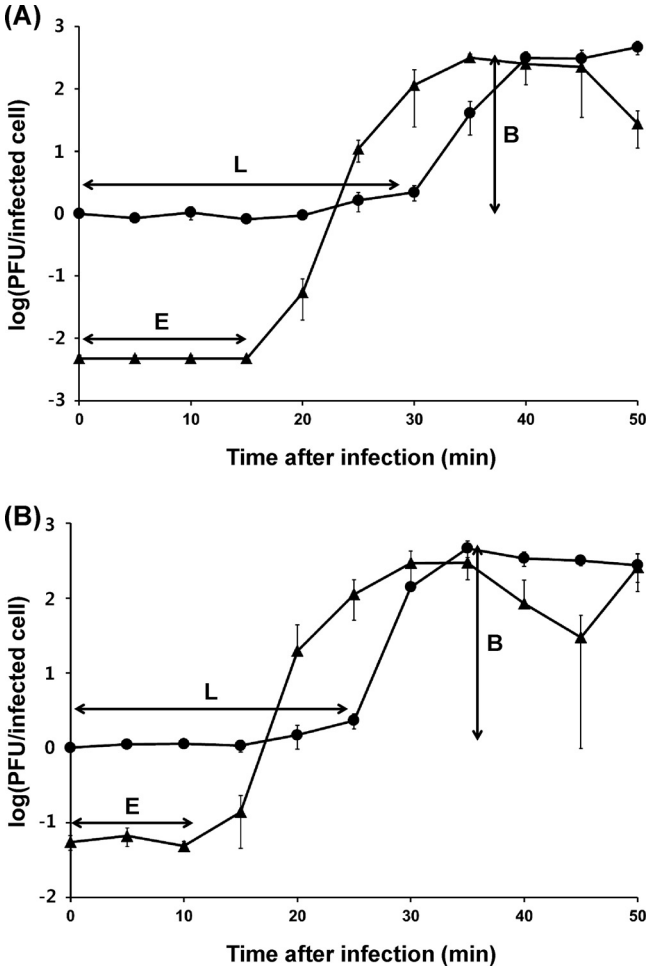

FIG 5 One-step growth curve analysis of phages SPN9CC (A) and SPN9CCM (B). Circles represent non-chloroform-treated samples, and triangles represent chloroform-treated samples. The error bars indicate the standard deviations of triplicate experiments. E, eclipse period; L, latency period; B, burst size.

lysis of *E. coli* host cells but the coexpression of endolysin and other proteins (endolysin plus holin or endolysin plus Rz/Rz1-like proteins) in *E. coli* host cells did result in host cell lysis, suggesting that endolysin may need support to cross the *E. coli* cytoplasmic membrane and that either holin or Rz/Rz1-like proteins could help endolysin to cross the membrane (Fig. 4B). It is intriguing that the main difference between the patterns of *E. coli* and *Salmonella* host cell growth inhibition by the SPN9CC lysis gene cluster is the role of Rz/Rz1-like proteins, which inhibit only *E. coli* host cell growth bacteriostatically (Fig. 4B).

Conversion of phenotypes in phage SPN9CC by deletion of the *cI* gene. CI, CII, and Cro are key proteins in the lysogeny control region (49–51). Among them, CI is a repressor causing termination of gene expression in the phage genome. Therefore, mutation of the *cI* gene can inhibit lysogen formation. The effects of *cI* gene deletion on the life cycle of phage SPN9CC was studied by constructing the Δ*cI* mutant phage by the BRED method (42). Interestingly, whereas phage SPN9CC generates distinct clear plaques with cloudy centers as lysogens, the Δ*cI* mutant phage SPN9CCM does not produce cloudy centers in the plaques, suggesting that the phenotype of Δ*cI* mutant phage may be converted from temperate to virulent (see Fig. S1C in the supplemental material). To further understand the plaque morphology change caused by *cI* deletion, one-step growth analyses and bacterial challenge assays of phages SPN9CC and SPN9CCM were compared. The one-step growth analyses revealed that while phage SPN9CC has relatively long eclipse and latency periods and a small burst size, phage SPN9CCM has much shorter eclipse and latency periods and a larger burst size (Fig. 5). The eclipse and latency periods of SPN9CC and SPN9CCM were 15 and 30 min and 10 and 20 min, respectively. The average burst sizes of phages SPN9CC and SPN9CCM were 220 and 280 PFU per cell, respectively, suggesting that the efficiency of phage multiplication was increased for SPN9CCM most likely because of an inability to form lysogens. Furthermore, bacterial challenge assays of phages SPN9CC and SPN9CCM with *S.* Typhimurium SL1344 demonstrated that the inhibition activity of phage SPN9CCM is much higher than that of phage SPN9CC (Fig. 6).

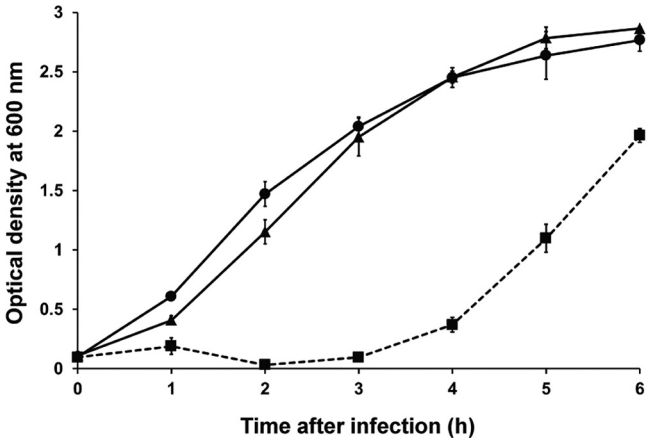

FIG 6 Assay of *S.* Typhimurium LT2C challenge with phages SPN9CC and SPN9CCM. Circles represent non-phage-treated samples, triangles represent SPN9CC-treated samples, and squares with a broken line represent SPN9CCM-treated samples. The SPN9CC- and SPN9CCM-treated samples were tested at a multiplicity of infection of 10.

DISCUSSION

Salmonellosis is one of the most common types of food poisoning caused by food-borne pathogens all over the world. To reduce this food poisoning, the bacteriophage approach has recently been appearing more attractive than antibiotic treatment because of the emergence of multidrug-resistant *Salmonella* strains (5, 6). To maximize the efficiency of this phage approach, further understanding of phage infection and host cell lysis mechanisms is required (15, 52). Phage P22 has been studied in the context of the development of a molecular transduction tool (18, 19), the identification of host cell specificity and a receptor (22, 23), the tail structure for host cell interaction (53, 54), the lysogeny control region (55, 56), superinfection exclusion (57, 58), and other areas. The P22-like phage group was previously proposed on the basis of the homology of virion assembly genes, which include those for ST104, ε34, ST64T, L, Sf6, c341, and HK620, among others (25). Recent improvement of genome sequencing and analysis technologies, such as NGS and bioinformatic tools, enabled us to analyze the full genome sequences of these P22-like phages and to study their characteristics at the genomic level. Recent comparative genomic analysis revealed that while their genomic characteristics are diverse, most likely because of horizontal gene transfer/exchange in the group, morphogenesis and DNA packaging are highly conserved (25, 26). However, the diversity of other genomic features may determine the specific characteristics of each phage in the group, such as host cell specificity, the lysogeny control region, and the host cell lysis system, involved in the mechanisms of host cell infection and lysis.

Salmonella-targeting temperate phage SPN9CC was isolated from a commercially processed broiler chicken skin sample, and its complete genome analysis suggests that phage SPN9CC is in the P22-like phage group. One-step growth analysis of phage SPN9CC revealed a longer latency period and a smaller burst size than those of other lytic phages, such as T7-like *Podoviridae* family phages (Fig. 5A) (59–61), suggesting that lysogen formation during phage infection may affect the host cell lysis activity of phage SPN9CC. A high frequency of observed mutants insensitive to this phage during a bacterial challenge test also supports this (see Fig. S2 in the supplemental material). Generally, superinfection of a lysogen by other phages is prevented by repression of the expression of superinfecting phage genes by CI repressor proteins by lysogen or host cell receptor modification (62). It is well known that the host cell receptor is modified once the host cell is lysogenized by phage (18, 26, 63). SPN9CC has LPS modification proteins homologous to GtrABC (SPN9CC_003, SPN9CC_002, and SPN9CC_001, respectively), which modify LPS to prevent superinfection of the SPN9CC lysogen. Furthermore, the resistance activity of the host cell lysogen caused by LPS modification during lysogenization may contribute to the formation of cloudy centers in SPN9CC plaques (see Fig. S1A). In the center of the plaques, a high phage concentration may promote lysogenization of phage SPN9CC, similar to phage P22 (64, 65) or the first lysogens formed may expand from the middle outward (see Fig. S1A).

The role of LPS modification proteins GtrABC and superinfection exclusion protein B is the prevention of other phage infections after lysogen formation via modification of the host cell O antigen of LPS (18, 19). Among the recombination proteins, the Abc1 and Abc2 anti-RecBCD proteins are involved in phage recombination and protect both ends of the linear phage genome from host cell RecBCD exonuclease and Erf recombination protein circularizes this linear genome by the ligation of both ends of the phage genome (66). Lysogeny control and antitermination determine the phage lytic/lysogenic cycles, depending on the host cell status. Replication proteins are produced during early gene expression, and they are responsible for phage genome replication. However, the functions of the *ea* and *nin* genes are unknown (67). Host cell lysis proteins such as holin, endolysin, and Rz/Rz1-like proteins have been suggested to cooperate in bursting the host cell after replication and reconstruction of the phage (21). Holin creates pinholes in the host cell inner membrane, and the subsequent secretion of endolysin via these pinholes results in host cell lysis. Although *ant* moron regions have been found in P22-like phages, these regions are highly variable among them (26) and the function of the *ant* moron is not clearly understood. Phage SPN9CC also has only one gene in this region, a *mnt* gene encoding a repressor protein, which is very similar to phages ST104 and ST64T. This Mnt repressor has been suggested to control the expression of *ant* gene encoding an antirepressor (68). As with other P22-like phages in the *Podoviridae* family, phage SPN9CC has only a tailspike protein without a tail fiber protein. This tailspike protein is homologous to other tailspike proteins observed in certain P22-like phages that target *S.* Typhimurium.

The complete genome sequence of phage SPN9CC and comparative genomic analyses with other P22-like phages revealed a diversity of phage infection and host cell lysis mechanisms in the group (Fig. 1 and 3). P22-like phages are in the family *Podoviridae* and have short tails, indicating that the tailspike protein is a major determinant of host specificity in P22-like phages (23, 69, 70). However, the tailspike protein is variable in the group, suggesting that the host specificity and host range of P22-like phages could be variable. Whereas phages P22, ST104, and ST64T with homologous tailspike proteins infect *S.* Typhimurium, phages ε34 and Sf6 with different types of tailspike proteins infect *S.* Anatum and even *Shigella*, respectively, supporting the notion of variable host range and specificity (25, 44, 71). Comparative analysis of the lysogeny control regions of P22-like phages indicated that the region of SPN9CC is nearly identical to that of phage ε34 but quite different

from that of phage P22, suggesting that P22-like phages may have diverse lytic/lysogenic decision mechanisms (Fig. 3B).

Characterization of the host cell lysis gene cluster of SPN9CC is important to understanding the host cell lysis mechanism of SPN9CC. The host cell lysis gene cluster encodes putative holin, endolysin, and Rz/Rz1-like proteins. This gene cluster of phage SPN9CC is quite different from those of P22 and phage ε34s but very similar to those of ST104 and even the *E. coli* K-12 DLP12 prophage, suggesting the possibility of *E. coli* cell lysis via the activity of lysis proteins that are encoded in the gene cluster of phage SPN9CC. The expression of these genes individually or in combinations in *S.* Typhimurium or *E. coli* host cells revealed that holin is a key protein for the lysis of the cells of both hosts, but endolysin could not achieve lysis by itself (Fig. 4A and B). These results indicate that endolysin of SPN9CC requires holin to cross the cytoplasmic membrane to act on the peptidoglycan in the periplasm. Rz/Rz1-like proteins are known accessory proteins of endolysin (21), and Rz/Rz1-like proteins alone or in combination with endolysin in *S.* Typhimurium did not exhibit cell lysis activity. Interestingly, Rz/Rz1-like proteins alone and even in combination with endolysin resulted in growth inhibition of *E. coli* even though the degree of inhibition was relatively low. However, the lysis activity of Rz/Rz1-like proteins in *E. coli* host cells is not fully understood. Comparative functional analysis of the *S.* Typhimurium and *E. coli* host cell lysis gene clusters revealed that this lysis protein combination works better in *E. coli* than in *S.* Typhimurium (Fig. 4A and B).

Comparison of host cell lysis activities by the bacterial challenge assay revealed that phage SPN9CCM had higher host cell lysis activity than phage SPN9CC. However, an SPN9CCM-resistant strain emerged 4 h after infection (Fig. 6). The phage adsorption assay conducted with the SPN9CCM-resistant strain indicated that more than 98% of phage SPN9CCM adsorbed to the wild-type *S.* Typhimurium host in 10 min but less than 5% of phage SPN9CCM adsorbed to the SPN9CCM-resistant strain under the same conditions (data not shown), suggesting that the host receptor for phage infection may be modified. Various LPS modification mechanisms (72) are known, and the exact growth recovery mechanism of SPN9CCM-infected host strains needs to be elucidated in the future.

In this study, comparative analysis of phage SPN9CC and P22-like phages provided novel insights into phage infection and *S.* Typhimurium host strain lysis mechanisms. We therefore believe that this study contributes to a better understanding of the new approach to bacteriophage treatment to inhibit food-borne pathogens, as well as to the development of newly optimized phages for therapy.

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