Systemic method to isolate large bacteriophages for use in biocontrol of a wide-range of pathogenic bacteria

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Large phages are characterized by genomes around 200 kbp or more. They can infect wide host ranges of bacteria and maintain long-lasting infection. There is no standard method for selective isolation of large phages. In this study, we developed a systemic method to isolate large phages and succeeded in isolating 11 large phages, named *Escherichia* phage E1~E11. Electron microscopy observations revealed typical *Myoviridae* phages with big capsids and long contractile tails. Genome sizes of the isolated phages were determined by pulsed-field gel electrophoresis and found to be in two groups, those around 200 kbp for E1, E2, E5, E6, E7, E9 and E10 phages, and others of approximately 450 kbp for E3, E4, E8 and E11 phages. The isolated large phages had wide host ranges: for example, E9 was effective against *Shigella sonnei* SH05001, *Shigella bydii* SH00007, *Shigella flexneri* SH00006, *Salmonella enterica* serovar Enteritidis SAL01078 and *Escherichia coli* C3000 (K-12 derivative), as well as its original host *E. coli* BL21. Screening of these jumbo phages was performed with non-pathogenic *E. coli* strains as hosts. Therefore, this method opens a way to isolate jumbo phages infecting wide ranges of pathogenic bacteria in a typical laboratory with standard laboratory strains as the hosts. The isolated large phages will be good candidates for biocontrol of various pathogens.

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Bacteriophages (phages) have been used for clinical applications since their first discovery at the beginning of the 20th century (1,2). The most important factors in evaluating phages as effective biocontrol agents are their host ranges and ability to execute lasting infection (3). Most bacteriophages have specific host ranges, usually limited to strains of the same bacterial species (3,4). However, good candidates for phage therapy can infect a broad range of pathogenic bacteria. To assure a broad host range of phage formulations against target bacteria, different types of phages are mixed as so called 'phage cocktails'. Unfortunately, there are many limitations of phage cocktail application associated with sustainable and continuous supply of medical and commercial formulations. Also, formulations containing too many phages can result in high manufacturing and development costs (5).

Jumbo phages are tailed phages characterized by a large genome (>200 kbp) and most of them are members of the family *Myovirida* (6). Most jumbo phages have multiple genes for expressing DNA polymerases and RNA polymerases (RNAPs) (7,8). It was reported that the expression of jumbo phage genes is independent of the host RNAP and may be driven solely by the phage RNAPs (9,10). Moreover, jumbo phages have genes for

enzymes involved in host cell lysis, such as endolysin, chitinase, glycoside hydrolase and lyases, that facilitate infection (11). Many other genes encoded by jumbo phages may function to reduce their dependence on bacterial hosts (12). Because of these special characteristics of jumbo phages, it is thought that most have a broad host range because of their reduced dependence on their host bacterium (11). In addition to this, some jumbo phages were reported to execute sustainable, long-lasting infection (13,14).

Despite the amazing characteristics of jumbo phages, only ~100 jumbo phages have been isolated since the first discovery of phage at the beginning of the last century. However, they may be more frequent and abundant in our environment, with a high impact on microbial ecology. Most jumbo phages have been isolated by chance by normal methods for bacteriophage propagation. An example is bacteriophage G, which was accidentally found during preparation of another bacteriophage (15). The inaccessibility of jumbo phages is attributable to our dependence on the classical procedures for phage detection and isolation (11,16). For example, using high top agar gel concentrations hinders the diffusion of jumbo phages and hence limits detection of their plaques (17). Removal of bacterial contamination by filtration could eliminate most jumbo phages coexisting in the sample (18). Unfortunately, there is still no standard method for isolation of jumbo phages. The aim of this study was to develop a method for selective isolation of

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large phages such as jumbo phages (with a genome larger than 200 kbp) and phages with a genome around 200 kbp including T4-like phages.

MATERIALS AND METHODS

Bacterial strains, bacteriophages and culture conditions   Table 1 lists bacterial strains used in this work. They included three strains of gram-positive bacteria from two genera (*Bacillus* and *Staphylococcus*) and 26 strains of gram-negative bacteria from 10 genera (*Acinetobacter, Escherichia, Klebsiella, Proteus, Providencia, Pseudomonas, Salmonella, Serratia, Shigella* and *Vibrio*). Bacteria were cultivated in LB medium (19) at appropriate temperatures for each strain.

Isolation of bacteriophages (special method for large phage isolation)
Sewage samples were collected from the Higashi-Hiroshima Wastewater Treatment Plant in Taguchi (Higashi-Hiroshima, Japan) at different stages of processing. A 400 ml portion of each sample was centrifuged at $5000 \times g$ for 10 min at room temperature to remove most particulates, including unwanted debris and microbial cells. After recentrifugation of the supernatant at $15,000 \times g$ for 1 h at 4°C, all the pellets were suspended by vortexing in 5 ml of SM buffer (50 mM Tris–HCl [pH 7.5], 100 mM NaCl, 10 mM $MgSO_4$, and 0.01% gelatin). To selectively remove small phages, this centrifugation–resuspension cycle was repeated three times. An equal volume of chloroform was added to the final suspension to kill any residual contaminating bacteria.

Selective detection of large phages   To facilitate detection of large phages, standard plaque assays were modified as follows: Bacterial cultures (*Escherichia coli* strains BL-21, XL-1-Blue, DH5α or Mach-1) were prepared in 4.5 ml LB broth adjusted to $OD_{600} = 0.25$. A 100 μl phage sample was mixed with 250 μl bacterial culture and stood for 10 min at room temperature to allow adsorption. With 4.5 ml of molten LB top agar (0.35%), the mixture was poured onto an LB agar plate (1.5% agar). The plates were incubated for 24 h at 23°C (or 18°C if strain Mach-1 was the host). Small plaques that appeared on the plates were selectively picked for further purification and enrichment. Single plaque purification was repeated three times to confirm the plaque was derived from only one kind of phage. Enriched phage preparation was obtained from plate lysates. The scheme of this systemic detection and isolation of large phages is summarized in Fig. 1.

Host range tests   The host range of phages was determined initially by spot tests and then standard plaque-forming assays (20). Test strains were mainly chosen from pathogenic bacteria (Table 1). Overnight cultures of bacterial strains prepared in LB medium were subcultured in 500 μl LB broth and adjusted to $OD_{600} = 1$. An aliquot (100 μl) of each bacterial subculture was mixed with 3 ml of molten LB top agar (0.35%), poured onto the surface of an LB agar plate (1.5% agar) and left to dry for 10 min. Then, 1 μl of each phage preparation (with titer $\sim 10^8$ plaque forming units/ml) was spotted onto the bacterial overlay (20), left for 15 min to

dry, and then incubated at 30°C for 24 h. When a lysis zone appeared, efficiency of plating was determined for each strain as the host (20).

Electron microscopy for initial phage characterization   High titers of phages were obtained from liquid cultures of *E. coli* BL21 as the host. Phage particles were purified by sucrose gradient (20–40%) centrifugation at $40,000 \times g$ for 1 h. Then, the concentrated phages were subjected to electron microscopic observation according to Ackermann (21). Briefly, phage particles were spotted onto a copper grid coated with formvar-carbon, left to adsorb for 2 min, stained with 1% Na-phosphotungstate, and observed under a Hitachi H600A electron microscope.

Pulsed-field gel electrophoresis for genomic analysis   The phage genome size was determined by pulsed-field gel electrophoresis (PFGE), as described by Higashiyama and Yamada (22). Briefly, after purification of phage particles by sucrose gradient (20–40%) centrifugation at $40,000 \times g$ for 1 h, they were embedded in 1.7% low-melting-point agarose (InCert agarose; FMC Corp., Philadelphia, PA, USA). Phage-containing plugs were treated by proteinase K (1 mg/ml; Merck Ltd., Tokyo, Japan) and 1% sarkosyl, and subjected to PFGE by a using a CHEF Mapper electrophoresis apparatus (Bio-Rad, Hercules, CA, USA).

Isolation and characterization of genomic DNA from phage particles   Standard techniques for DNA isolation and digestion with restriction enzymes and other nucleases, including nuclease S1were followed according to Sambrook and Russell (19).

RESULTS

Isolation of *Escherichia* large phages   From our experience in isolation and characterization of jumbo phages infecting *Ralstonia solanacearum* (13,23), we chose five conditions to select large phages infecting pathogenic enteric bacteria: (i) Contaminating microbial cells were killed by chloroform without membrane filtration. (ii) Relatively large phage particles were selectively precipitated by differential centrifugation. (iii) Plaque assays used a low concentration of top agar (0.35%). (iv) Small plaques were selectively picked since large phages always form very small plaques. (v) Plaque assays were conducted at lower temperatures to delay host growth. The last step was important to make minute plaques clearly visible. The effectiveness of this method is shown in Fig. 2 compared with a standard method (with 0.7% top agar and incubated at 37°C).

By using these approaches (as described in Materials and methods), we started isolation of large phages from sewage

TABLE 1. Host range of *Escherichia* phages on gram-negative and gram-positive bacterial strains.

E11	E10	E9	E8	E7	E6	E5	E4	E3	E2	E1	Host (bacterial species)
−	−	−	−	−	−	−	−	−	−	−	*Acinetobacter baumannii* 395
+	+	+	+	+	+	+	+	+	+	+	*Escherichia coli* BL21
−	−	+	+	+	+	+	−	−	+	+	*E. coli* C3000 (C strain)
−	−	−	−	+	−	−	−	−	+	+	*E. coli* K12
−	−	−	−	−	−	−	−	−	−	−	*E. coli* O157:H7
−	−	−	−	−	−	−	−	−	−	−	Enterohaemorrhagic *E. coli* EHEC 03064
−	−	−	−	−	−	−	−	−	−	−	*E. coli* 7
−	−	−	−	−	−	−	−	−	−	−	*Klebsiella pneumoniae* 63
−	−	−	−	−	−	−	−	−	−	−	*Proteus mirabilis* 59
−	−	−	−	−	−	−	−	−	−	−	*Providencia stuartii* 50
−	−	−	−	−	−	−	−	−	−	−	*Pseudomonas aeruginosa* 16
−	−	−	−	−	−	−	−	−	−	−	*Pseudomonas aeruginosa* PAO1
−	−	−	−	−	−	−	+	+	−	−	*Salmonella enterica* serovar Paratyphi B SAL 04100
−	−	+	−	−	−	−	+	−	−	−	*Salmonella enterica* serovar Enteritidis SAL01078
+	+	+	−	+	+	+	−	−	+	+	*Shigella sonnei* SH05001
+	−	+	−	+	−	−	−	−	+	+	*Shigella boydii* SH00007
+	+	+	−	+	−	−	−	−	−	+	*Shigella flexneri* SH00006
−	−	−	+	−	−	−	+	+	−	−	*Serratia marcescens* D601
−	−	−	−	−	−	−	−	−	−	−	*Serratia marcescens* (ATCC 13880)
−	−	−	−	−	−	−	−	−	−	−	*Vibrio cholerae* O139 MDO6
−	−	−	−	−	−	−	−	−	−	−	*Vibrio cholera* P1418O1 E1
−	−	−	−	−	−	−	−	−	−	−	*Vibrio fluvialis* AQ0005
−	−	−	−	−	−	−	−	−	−	−	*Vibrio parahaemolyticus* ACA339
−	−	−	−	−	−	−	−	−	−	−	*Bacillus subtilis* 168
−	−	−	−	−	−	−	−	−	−	−	*Staphylococcus aureus* ATCC 6538
−	−	−	−	−	−	−	−	−	−	−	*Staphylococcus epidermidis* ATCC 35984

Sensitivity: +, sensitive (EOP $> 10^{-2}$ PUF/plate); −, resistant (EOP $< 10^{-6}$ PFU/plate). An EOP of 1 was equivalent to 408, 374, 280, 265, 355, 334, 369, 240, 530, 439, and 382 PFU per plate for E1~E11 with *Escherichia coli* BL21 as the host, respectively.
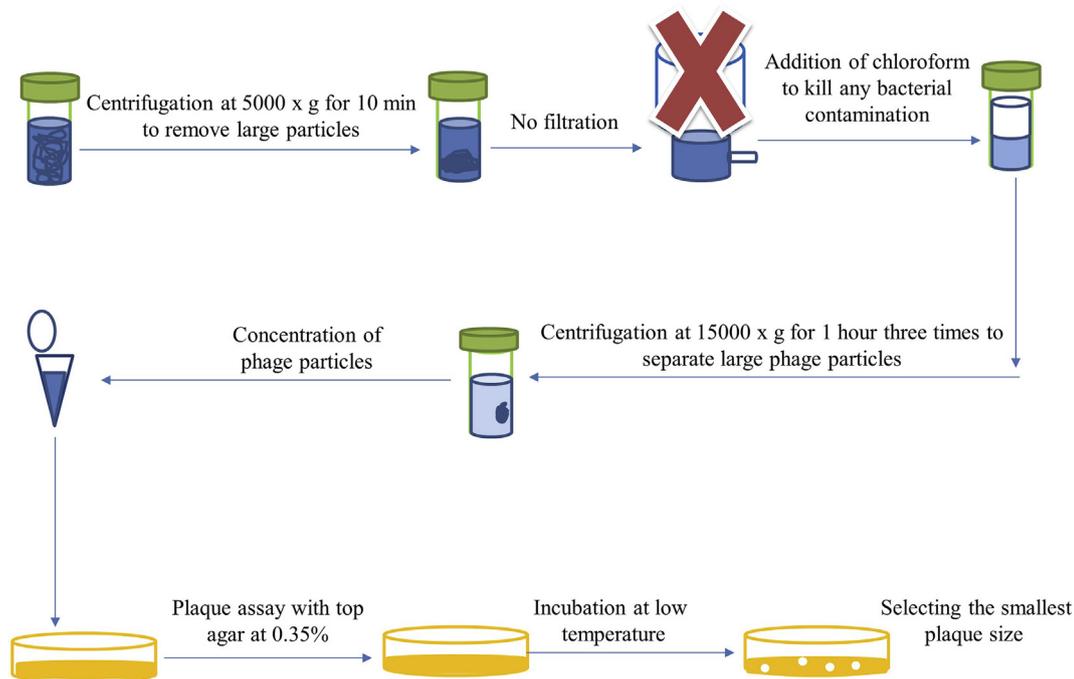
FIG. 1. Systemic detection and isolation of large phages. After treating with chloroform (without filtration), relatively large phage particles were separated by differential centrifugation. Plaque assays using low concentrations of top agar (0.35%) allow large phage particles to diffuse easily. Plaque assays are conducted at lower temperatures to delay host growth, resulting in larger plaque formation by phages. Smaller plaques are picked, as large phages always form very small plaques.

samples collected from a large wastewater treatment plant. Eleven candidate phages (E1~E11) were finally obtained, which stably formed very small plaques (<0.1 mm on 0.35% top agar). *E. coli* BL21 was the ideal host strain for screening E1~E11 phages. Around 30 ± 5 plaques appeared on the first assay plates and the level of small plaques was about 16%. For comparison, no such small plaques were obtained by a conventional method with filtration and plaque assays with 0.7% top agar.

Electron microscopy observation   Electron microscopic observation of phages E1~E11 revealed characteristic features of the family *Myoviridae* (Fig. 3). There are two types of particles: (i) T4 like particles characterized by a prolate head, a contractile tail, and long tail fibers (E1, E2, E5, E7, E9 and E10); (ii) large particles
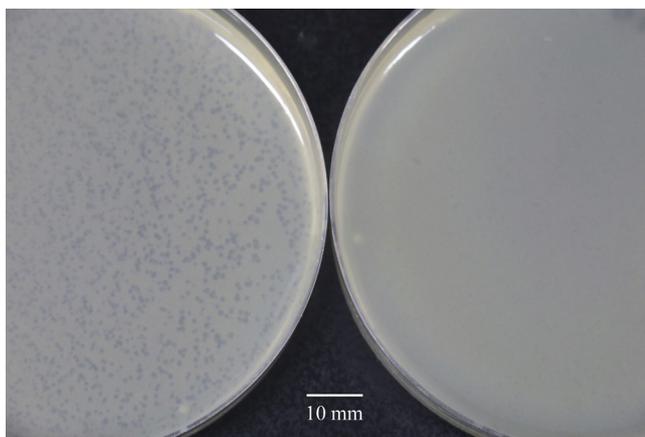

FIG. 2. Comparison of phage plaques formed by the new method (schematically shown in Fig. 1) compared with those by a standard method. Relatively large clear plaques are visible for phage E8 with *Escherichia coli* BL21 as the host by the new method (left) but plaques are very obscure by a standard method (the same phage and host with 0.7% top agar and incubated at 37°C) shown on the right.
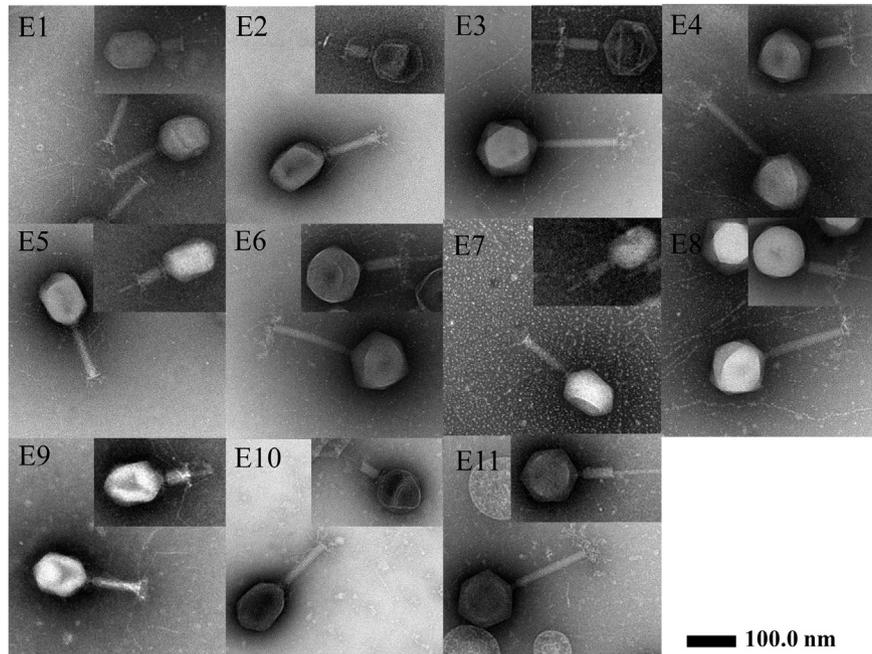
characterized by an icosahedral head (diameter 110 ± 5.5 nm) and a long contractile tail (length 145 ± 7.25 nm) (E3, E4, E6, E8 and E11).

Genomic analysis by PFGE   To determine genomic DNA sizes of the phages, we performed in-gel PFGE with purified phage particles. Fig. 4 shows the DNA separation patterns of these phages. The genomes of phages E1, E2, E5, E6, E7, E9 and E10 were around 200 kbp. While genome sizes of E3, E4, E8 and E11 were about 450 kbp. These sizes in PFGE were not affected by S1 nuclease digestion (23), indicating linear DNA forms of these genomes. *Eco*RV digestion patterns of genomic DNAs of E1~E11 are shown in Fig. 5A, where E1, E2, E6, E7, and E10 showed digested banding patterns, whereas E3, E4, E5, E8, E9, and E11 DNAs were not digested. Although large fragments for E1, E2, and E6 DNAs were not well separated, the digested fragmentation patterns were different from each other among the ~200-kbp genome phages. As for ~450-kbp genome phages, all E3, E4, E8, and E11 DNAs were digested with *Hae*III (Fig. 5B), giving banding patterns similar to each other among E3, E4, and E8 with some differences. E11 DNA gave a unique banding pattern. Other restriction enzymes we tried failed to digest these large DNA genomes, suggesting some modification of the genomic DNA. Although exact genomic size for these phages could not be determined, 11 phages isolated in this study were large (putative jumbo) phages by the criterion of genome size ~200 kbp.

Host range of the phages   Host ranges of phages E1~E11 were determined with various bacterial strains (Table 1). E9 phage has a wide host range, infecting *Shigella sonnei* SH05001, *Salmonella enterica* serovar Enteritidis SAL01078 and *E. coli* C3000 (K-12 derivative), as well as its original host *E. coli* BL21. The most of other *Escherichia* phages could lyse multiple bacterial hosts, including *S. sonnei* SH05001, *Shigella boydii* SH00007, *Shigella flexneri* SH00006, *E. coli* C3000 and *E. coli* BL21. These results indicate that the large phages isolated using *E. coli* laboratory strains as hosts have a wide host range including important pathogens. This is the

FIG. 3. Morphology of eleven Escherichia phages (E1～E11) stained with 1% Na-phosphotungstate and examined by transmission electron microscopy. All phages are members of the family *Myoviridae*. Six phages (E1, E2, E5, E7, E9, and E10) are typical *Caudovirales* phages characterized by a prolate head and contractile tail. The remaining five phages (E3, E4, E6, E8, and E11) showed large particles characterized by an icosahedral head and long contractile tail without visible tail fibers. A contracted tail is shown in each inlet.

first demonstration that large phages infecting wide ranges of pathogenic bacteria can be screened in normal laboratories using nonpathogenic bacterial strains as the hosts.

DISCUSSION

By making some modifications to the classical methods for detection and isolation of bacteriophages, we could selectively obtain large phages. The modifications included: (i) Initial selection by differential centrifugation of samples to precipitate relatively large particles, and killing of contaminating bacteria with chloroform without filtration. This allowed us to remove small phages but retain large phage particles without contamination from small bacterial cells. (ii) Plaque assays using low concentrations of top agar (0.35%) and picking of smaller plaques, as large phages always form very small plaques. In the top
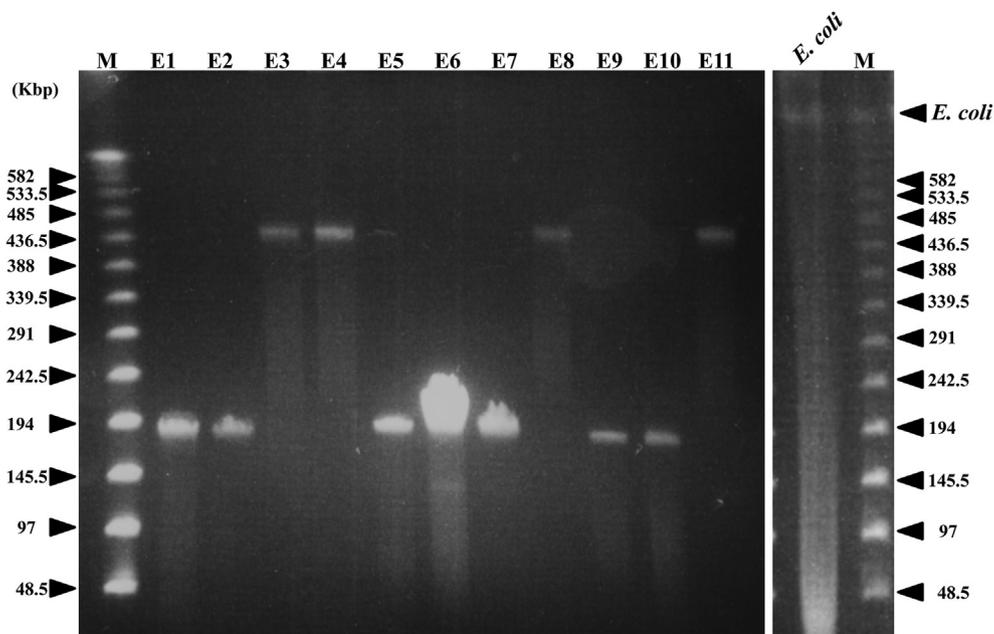

FIG. 4. Pulsed-field gel electrophoresis (CHEF) separation patterns of the genomic DNAs of 11 Escherichia phages (E1～E11). The genomic size of seven phages (E1, E2, E5, E6, E7, E9, and E10) is around 200 kbp, whereas that of the other four phages (E3, E4, E8 and E11) is around 450 kbp. For comparison, the genomic DNA of host *E. coli* cells (strain BL21) separated under the same condition is shown on the right side. M, lambda DNA ladder for size markers.
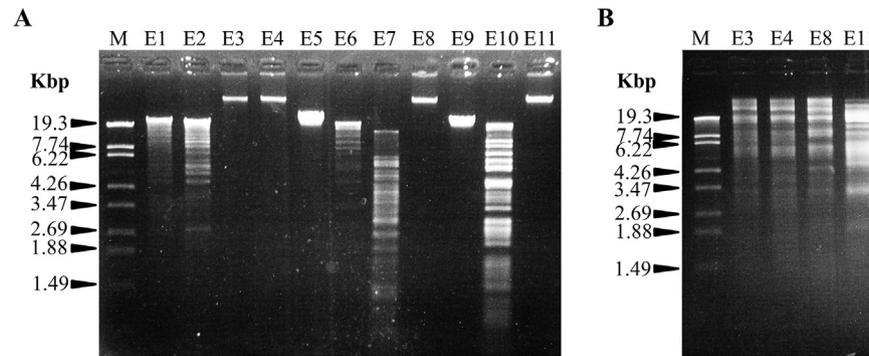
FIG. 5. Restriction enzyme digestion patterns of genomic DNA of Escherichia phages. (A) Eleven genomic DNAs were digested with *Eco*RV. (B) Genomic DNAs of E3, E4, E8, and E11 were digested with *Hae*III. M, molecular marker (Lambda-*Sty*I fragments).

agar of ∼0.7% usually used in the standard method, large phage particles cannot form visible plaques because of limited diffusion. Lowering the top agar concentration means phage particles can diffuse more easily. However, even in this condition, plaques of large phages are small. (iii) Plaque assays were conducted at lower temperatures to delay host growth, resulting in larger plaque formation by phages. With *E. coli* strains as the host, our preliminary tests of plaque formation by lambda phage gave the largest plaques at 23°C. Vigorous growth of the host strain at higher temperatures (e.g., 37°C for *E. coli*) leads to rapid spread of cells over the plate surface and inactive states of the cells. Small plaques of large phages might be caused by other factors such as small burst size, low activity of lytic enzymes etc, but by using this systemic method, large phages easily missing from ordinary screening can be efficiently detected and isolated as shown in Fig. 2. However, even by using this method, some large phages may not be obtained because of their sensitivity to chloroform (24,25). For such phages, further refinement including extended differential centrifugation without chloroform treatment should be given to the method.

We used laboratory strains of *E. coli* as the hosts and obtained large phages (including T4-like phages) that showed wide-host ranges, including important pathogens such as strains of *Shigella* and *Salmonella*. Phages are usually species-specific and even strain-specific (3). However, some polyvalent phages infecting strains of either different genera or species have been reported, predominantly among phages of *Enterobacteria* (26,27) and *Staphylococcus* (28). For example, Shigella phage SH7 was reported to infect strains of *E. coli*, *Salmonella* Paratyphi, and *Shigella dysenteriae* in addition to its original host *Shigella flexeri* (26). In respect of host strains ranging over three different genera including *Escherichia*, *Shigella*, and *Salmonella*, phage E9 found in this work is similar to SH7. Phage E4 also showed a unique host range covering three genera such as *Escherichia*, *Salmonella*, and *Serratia*. We found such a rare case of host range reported for bacteriophage χ (29). These phages may be effectively used for biocontrol of pathogenic enteric bacteria solely or in phage cocktails.

Handling of usual pathogenic bacterial strains in biosafety level 2 or 3 laboratories requires special experimental facilities, skills, and care. However, the screening method adapted here for large phages used biosafety level 1 hosts. It is thought that large phages with many adaptive genes generally have wide host ranges (23,30−32) so that large phages will be good candidates for various biocontrol purposes. Long-lasting phage effects also increase the potential value of large phages (14). We hope this method will open a new door to extend jumbo phage isolation and their use in biocontrol of a wide range of pathogenic bacteria.

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