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Functional characterization of a novel lytic phage EcSw isolated from *Sus scrofa domesticus* and its potential for phage therapy

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ABSTRACT

In this study, multi-drug resistant *Escherichia coli* Sw1 (*E. coli* Sw1) and active lytic phage EcSw was isolated from feces samples of *Sus scrofa domesticus* (piglet) suffering from diarrhea. Transmission electron microscopy (TEM) indicated that isolated EcSw belongs to the *Myoviridae* family with an icosahedral head (80 ± 4) and a long tail (180 ± 5 nm). The EcSw phage genome size was estimated to be approximately 75 Kb of double-stranded DNA (dsDNA). Phage dynamic studies show that the latent period and burst size of EcSw were approximately 20 min and 28 PFU per cell, respectively. Interestingly, the EcSw phage can tolerate a wide range of environmental conditions, such as temperature, pH and ions (Ca^{2+} and Mg^{2+}). Furthermore, genome sequence analysis revealed that the lytic genes of the EcSw phage are notably similar to those of enterobacteria phages. In addition, phage-antibiotic synergy has notable effects compared with the effects of phages or antibiotics alone. Inhibition of *E. coli* Sw1 and 0157:H7 strains showed that the limitations of host specificity and infectivity of EcSw. Even though, it has considerable potential for phage therapy for handling the problem of the emergence of multidrug resistant pathogens.

1. Introduction

Escherichia coli strains such as O157:H7 are the most significant pathogen of humans and animals in the world [30], which is highly virulent with a low infective dose (10–100 cells), with a tremendous threat to public health and is the cause of significant economic losses worldwide [27]. *E. coli* 0157:H7 has developed strong resistance against many antibiotics that are used in human and veterinary medicine [33]. Health risks have increased for humans due to the use of antibiotics in livestock that has resulted in the emergence of antibiotic-resistant bacteria [24]. Antimicrobial use in swine may be a factor in the emergence of antimicrobial resistance in *E. coli* O157 [30]. Even a low dose of antibiotics in feed used in pig industries, increases the diversity of antibiotic resistance genes [21]. Furthermore, there is currently no animal vaccine available against *E. coli* 0157:H7 [32].

Bacteriophages are a promising alternative to antibiotics for the reduction of pathogens [37]. Continuing efforts are required to isolate novel phages with a broad host range to control or reduce the prevalence of antibiotic resistant *E. coli* 0157:H7 strains [32]. Several reports have been published on the isolation of phages that are specific to pathogenic *E. coli* from feces of humans and other animals [26]. It is known that phage therapy can be limited by a narrow host range of the phage [20]. This can be overcome by a phage cocktail treatment or by the combination of several phages, because there are no negative effects of phage activity on the host animal [27]. Minimizing the use of antibiotics is the recommended strategy to avoid the rapid emergence of antibiotic resistant bacteria [17]. The phage antibiotic combination may have a positive effect on increasing the effectiveness of the treatment and on decreasing the amount of antibiotic that is administered [7,9].

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The control of infectious diseases that are caused by drug-resistant bacteria requires alternatives to conventional antibiotics [25]. Phage therapy has been considered as a potential bio-control approach for antibiotic resistant bacteria [2]. A virulent phage can infect and lyse host bacteria efficiently, and the development of phage resistance is less common than the development antibiotic resistance [19]. Reducing harmful and pathogenic bacteria in the environment and in animals has been considered to be the main target of phage therapy [31]. Most virulent phages can inhibit the growth of the target bacterium with high specificity, and they can do so as efficiently as commonly used antimicrobial agents [18]. Therefore, phages are becoming popular natural alternatives to antibiotics for controlling pathogenic bacteria [37,40].

In this study, we isolated the virulent lytic phage EcSw, which is specific to multi-drug resistant *E. coli* Sw1 from feces of piglets suffering from diarrhea. We investigated the phage dynamics of infective characteristics (burst size, latent period and optimal multiplicity of infection), host range, stability (temperature, pH and ions), genome size, sequence analysis of phage lytic genes and the potential for combining the phages with antibiotics for efficient control of drug resistant *E. coli* strains.

2. Materials and methods

2.1. Bacterial strains and culture conditions

E. coli strains were isolated from feces sample of piglets infected with diarrhea. These were from a swine farm (Incheon, South Korea). The samples (1 g) were serially diluted and spread on LB agar supplemented with ampicillin to recover an ampicillin resistant bacterium. After overnight incubation at 37 °C, representative colonies were inoculated and spread on fresh LB agar supplemented with ampicillin for purification. For identification of the bacterial strain, sequencing analysis was done by using two universal bacterial primers, 27F (5′- AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTACCTTGTTACGACTT-3′), to amplify the 16s ribosomal RNA gene of the host bacterium. To identify *E. coli* Sw1, the sequence report was analyzed with Basic Local Alignment Search Tool (BLAST). The results confirmed that the bacteria were closely related to *E. coli* 0157:H7 and *Shigella sonnei* Ss046. Host bacterium was treated with Kovac's reagent (25 ml of concentrated HCl, 75 ml of Amyl alcohol and 5 g of paradimethylamino-benzaldehyde) to differentiate *E. coli* and *Shigella* strains. Isolated bacterial strains were stored at −80 °C in 40% glycerol solution. All of the bacterial strains were cultured in their specific media and conditions. These are listed in Supplemental Table A.1.

2.2. Isolation and purification of EcSw

For phage isolation, 5 g of piglet feces were mixed with 45 ml of LB broth, and incubated with 1% overnight cultured *E. coli* Sw1 strain for 16 h at 37 °C. After centrifugation (3500 × g, 15 min), the supernatant was collected and filtered through a 0.22 μm syringe filter (Corning, New York, USA). To confirm the presence of phage in the filtrate, soft agar (per liter) (LB broth, 25 g; agar, 0.4%; NaCl, 5 g; $MgSO_4$ $7H_2O$, 0.2 g; $CaCl_2$, 0.15 g; pH 7.5) was mixed with 200 μl of overnight cultured *E. coli* Sw1 and 100 μl of filtrate. This mixture was poured on pre-solidified LB agar (1.5% agar). Plaque formation was monitored after overnight incubation at 37 °C. For purification of the phage, a single plaque was picked up with a sterile tip and eluted in 1 ml of sodium chloride - magnesium sulfate (SM) buffer (100 mM NaCl, 10 mM $MgSO_4$. $7H_2O$ and 50 mM Tris HCl, pH 7.5), followed by filter sterilization as described above. This process was repeated at least three times until consistent plaque morphology was obtained. To increase the concentration of EcSw, 200 μl of overnight culture and 100 μl of eluted phage were mixed with soft agar for the double agar overlay method [8,22]. The phage was plate-eluted with 10 ml of SM buffer, followed by centrifugation and filter-sterilization with a 0.22 μm syringe filter. The phage titer was determined by the double agar overlay method. A high titer stock of EcSw was obtained for further assays and stored at 4 °C.

2.3. Scanning electron microscopy (SEM)

Morphological images of the host bacterium *E. coli* Sw1 was obtained using Field Emission Scanning Electron Microscopy. The bacterial isolate was treated with EcSw at a Multiplicity of Infection (MOI) of 1 and treated or non-treated bacterial cells (10^7 CFU/ml) were centrifuged at 3000 × g for 30 min. The pellets were washed thrice with 1× phosphate buffered saline (PBS) and pre-fixed with 2.5% glutaraldehyde for 30 min at room temperature. The pre-fixed cells were washed twice with 1× PBS and dehydrated with 25, 50, 75 and 100% of ethanol. The fixed cells were air-dried and coated with osmium using ion sputter (E-1030, Hitachi, Japan). The pre-treated samples were observed by FE-SEM using secondary electron (SE) detectors with 5 kV acceleration voltage (S-4800, Hitachi, Japan).

2.4. Transmission electron microscopy (TEM)

For transmission electron microscopy, a high titer phage stock (10^{10} PFU/ml) was prepared, and a drop was placed on a copper grid coated with carbon (SPI Supplies, PA, USA). This was then negative stained with 1% uranyl acetate (pH 4.5) for 10–20 s. Residual liquid was blotted off immediately. Transmission electron micrographs were captured using a JEM-1400 plus electron microscope that was operated at 120 kV. The phages were measured from the TEM micrographs with magnifications of ×40,000 and 100,000.

2.5. Determination of phage host range

The bacterial strains (n = 24) used in this study included *E. coli* strains (KF918342, ATCC35150, K-12, BL-21 and $DH_5\alpha$), *Aeromonas* spp., (*Aeromonas salmonicida salmonicida*, *Aeromonas salmonocida masoucida* and *Aeromonas hydrophila*), *Salmonella* spp. (*Salmonella enterica enterica* and *Salmonella typhimurium*), *Vibrio* spp. (*Vibrio ichthyoenteri*, *Vibrio proteolyticus*, *Vibrio parahaemolyticus*, *Vibrio tapetis* and *Vibrio vulnificus*) and 9 additional strains of different genera; these strains are listed in Supplemental Table A.1. Overlays were inoculated with 200 μl of each bacterium, and poured on pre-solidified LB and TSB agar plate depending on the bacterial strains. Once the overlay was dry, 10 μl of phage stock (10^{10} PFU/ml) was spotted on the overlay. The plates were left to dry and incubated at 37 °C overnight.

2.6. Host cell lysis test

To determine the optimal MOI of EcSw, an overnight culture of *E. coli* Sw1 was inoculated with 10 ml of fresh LB medium and incubated at 37 °C at 150 rpm until the optical density (OD) value reached 0.2 at OD_{620}. EcSw stock (10^{10} PFU/ml) was then added (MOI - 0.01, 0.1, 1 and 10) to cultures, and incubated for 6 h. Bacterial growth was monitored by measuring the OD_{620} values at each hour.

2.7. Single-step growth curve

For the single step growth curve, the previously described method was used with minor modification [22]. Briefly, overnight