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In vitro bacteriolytic activity of *Salmonella* specific novel isolated bacteriophage

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ABSTRACT: *Salmonella* is an important agent that causes foodborne gastrointestinal infections. *Salmonella enterica* serovar Enteritidis, serovar Typhimurium, and serovar Infantis are the main pathogenic agents of several enteric infections worldwide. Due to the emergence of multidrug resistant *Salmonella* strains, there is a need for new administration strategies alternative to antibiotics, such as bacteriophages, for effective control of them. The main objectives of this study were to isolate and characterize *Salmonella* spp. bacteriophage and compare its activity to commercial phage product and assess the effect of bacteriophages in vitro during 24-h. Because of the study, it was found that the newly isolated and named vB_SiM_12 phage could lyse 100% of the strains tested. Bacterial growth with dilutions of vB_SiM_12 phage (MOI ranging from 0.001 to 10) was measured during 24-h. The optic density values of the samples with added bacteriophage remained lower than the control sample during the 24-h incubation period. The results show that using phages may offer a promising alternative to combat biological control agents against *Salmonella* infection.

KEYWORDS: Bacteriophage; bacteriolytic activity; invitro turbidity test; *Salmonella spp*..

1. INTRODUCTION

Salmonella is one of the crucial foodborne pathogens and is a zoonotic infection agent that causes a significant public health burden worldwide. They can be transmitted by cross-contamination of prepared and raw foods and can cause foodborne gastrointestinal infections [1, 2]. The main sources of contamination of *Salmonella*, which has many subspecies, are wild and domestic animals (farm animals, birds, rodents) and the intestinal tract of humans. *Salmonella* spreads to many places in nature with the faeces of animals, causing contamination of water and food, and poisoning and diseases are seen as a result of people consuming contaminated food [3]. *Salmonella* Typhi (*S.* Typhi) and *S.* Paratyphi infections are mostly caused by food or water contaminated with the faeces of sick or asymptomatic chronic carriers [4]. Gastroenteric disorders caused by *Salmonella* species are generally called Salmonellosis infections. Eggs, poultry, meat products, milk, and dairy products are the most common causes of Salmonellosis infections. When food contaminated with *Salmonella* is consumed, symptoms such as chills, vomiting, headache, nausea, abdominal pain, and diarrhea usually begin 12–36 hours later. The incubation period varies from 4 to 72 h. The disease lasts between 3 and 7 days [5, 6]. The symptoms are more severe, especially in young, old, pregnant, and immunocompromised individuals, threatening human life with the loss of water in the body [7].

The incidence and mortality rate of the disease varies from region to region, but the mortality rate can be up to 7% despite antibiotic treatment [1]. In a report by the European Food Safety Authority (EFSA) in the European Union in 2011, it was stated that *Salmonella* is the most recognized agent related to food-borne outbreaks. In the latest report of EFSA, Salmonellosis ranks second in foodborne infections [8]. The most common *Salmonella* serotypes in humans in this report are *S.* Enteritidis, *S.* Typhimurium, and *S.* Infantis have been reported. The decrease in typhoid cases in the world in the last century has been associated with an increase in the drinking water purification process, pasteurization process, and taking measures to avoid contact with faecal wastes in the food chain. Typhoid fever is no longer endemic in developed countries; however, infection with other serovars of *S. enterica,* often referred to as non-typhoid *Salmonella*, remains a major public health problem. The continued development of antibiotic resistance is a global concern due to

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the unnecessary inappropriate use of antibiotics in human health and agricultural practices and their use as a feed additive to prevent bacterial diseases in the farm [7, 9]. Therefore, due to the emergence of multidrug resistant *Salmonella*, there is a need for new alternative antibiotic administration strategies for effective control of *Salmonella* [10].

With the discovery of the lethal effect of bacteriophages on bacterial hosts, it has been evaluated as an alternative treatment option [11]. Bacteriophages can be used as bioprotectors against pathogens at every stage of the food chain. They can be used for the prevention or reduction of diseases in farm animals, disinfection of carcasses and raw products, disinfection of tools-equipment, and extending the shelf life of foods [12]. The most important advantages of bacteriophages are that they are cheap, easy to obtain, preserve the natural microbiota and are not toxic. In addition, phages show activity independent of antibiotic resistance [13]. Commercial preparations of phages against various pathogens have begun to be produced. In the food industry, Listex TM P100 produced by EBI Food Safety is used against Listeria in cheese and meat products. In 2006, the FDA consented to its use of Intralytix LMP 102 product against Listeria in ready-made meat and poultry yields [14, 15]. In recent years, it has been granted as a Generally Recognized as Safe (GRAS) substance by the FDA in some phage products (for example, SalmoFresh™ and PhageGuard S™). bacteriophages can be used effectively against foodborne pathogens with specific targets without changing the microbial ecology of the environment [15].

In recent years, the number of studies on the use of bacteriophages as biocontrol tool in food industry has increased significantly. Poultry is the most important source of contamination for *Salmonella* infections, which are critical among foodborne infections in humans [16]. Increasing antimicrobial resistance, especially in *Salmonella*, has led to an increased interest in alternative treatments for the prevention and control of this infection. Therefore, it was aimed to evaluate the potential usage of the newly isolated bacteriophage in vitro and investigate its lytic activity compared with commercial preparations in this study.

2. RESULTS

2.1. The morphological analysis of bacteriophage

One phage was successfully isolated from samples of river water located at 39.965142 and 32.858040 in Ankara, Turkey. The vB_SiM_12 phage formed small clear plaques (Figure 1).

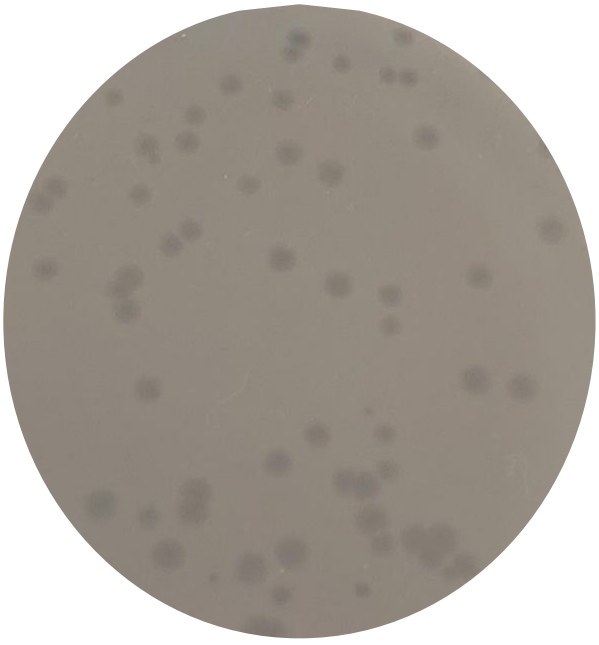

Figure 1. vB_SiM_12 phageplaquemorphology on plate.

Examination of phage particle morphology by TEM revealed that the size of the vB_SiM_12 phage head was 82.7 86.2 nm, and the size of the tail was 144.8 24 nm (Figure 2). It was considered to belong to the *Myoviridae* family.

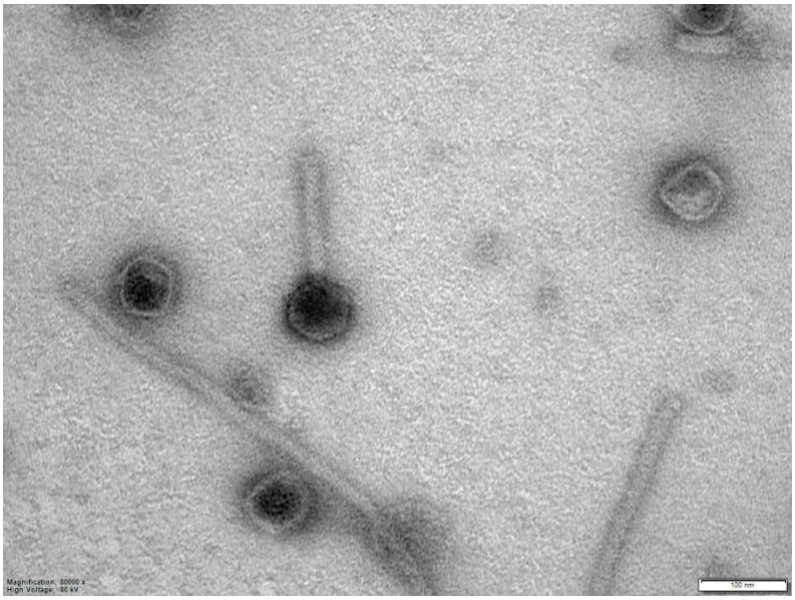

Figure 2. TEM micrograph of vB_SiM_12 phage. Morphology of phage was studied using a JEOL 100C electron microscope (Hitachi HT7800).

2.2. In vitro susceptibilities of bacteriophages

The activities of ENKO phage, INTESTI phage, and vB_SiM_12 phage on 25 *Salmonella* spp. were tested and the results are shown in Table 1. Their lytic activity was found to be 92%,88%, and 100%, respectively. The newly isolated laboratory vB_SiM_12 phage was found to have a lytic activity on all *Salmonella* spp.. It has been observed that vB_SiM_12 phage has strong activity on *Salmonella* spp. INTESTI phage showed the lowest lytic activity on strains. It was also found to be ineffective against *S.* Typhimurium strain.

2.3. In vitro turbidity assays

In a 24-h growth curve of bacterial sample treated with each bacteriophage at different MOI of 10, 1, 0.1, 0.01, 0.001 and there was a brief rise in OD 600 (up to ~2.5 to 3 h incubation) before drop-in turbidity compared to the untreated controls (Figure 3). The OD_{600} of all samples treated with bacteriophages remained well below that of the untreated controls throughout the 24-h incubation period. The lowest OD_{600} readings over the 24-h period after treatment with phage 0.01, 0.1, and 1 MOI were observed after 7h. Phage infection significantly decreased the bacterial culture turbidity in comparison with the control. However, an increase in turbidity (OD 600) was observed in MOI 0.001 and 10. It was observed that the efficacy of phage was weak at high MOI and very low MOI values. It is thought that it takes slightly longer to provide phage progeny for full efficacy at low MOI values. This increase in turbidity was most probably due to the growth of phage-resistant bacteria after ~2 h.

Table 1. Bacteriophage susceptibilities of 25 *Salmonella* spp. strains

No	Bacteria	ENKO	INTESTI	vB_SiM_12 phage
1	*S.* Infantis	-	-	+
2	*S.* Infantis	+	+	+
3	*S.* Infantis	+	+	+++
4	*S.* Infantis	-	-	+
5	*S.* Infantis	+	+	+++
6	*S.* Infantis	+++	+++	+++
7	*S.* Infantis	+	+	+++
8	*S.* Enteritidis	+++	+++	+++
9	*S.* Infantis	+	+	+++
10	*S.* Infantis	+	+++	+++

11	*S.* Infantis	+++	+++	+++
12	*S.* Infantis	+	+	+++
13	*S.* Infantis	+	+	+++
14	*S.* Infantis	++	++	+++
15	*S.* Infantis	+	+	+++
16	*S.* Infantis	++	+++	+++
17	*S.* Infantis	+++	+++	+++
18	*S.* Infantis	+++	+++	+++
19	*S.* Infantis	+++	+++	++
20	*S.* Infantis	++	+++	++
21	*S.* Infantis	+	+	+
22	*S.* Infantis	++	+	+
23	*S.* Infantis	++	+	+
24	*S.* Infantis	++	+	+
25	*S.* Typhimurium	+++	-	+
	Lytic effect (%)	**92**	**88**	**100**

+++: CL (Clear Lysis), ++: SCL (Semi-Clear Lysis), +: OL (Opaque Lysis), -: No lysis.

3. DISCUSSION

The United States Centers for Disease Control and Prevention (CDC) reported 1.35 million cases of Salmonellosis, with 26,500 hospitalizations and 420 deaths in the United States every year [17]. On farms, especially feed, soil, water, and faces are common sources of *Salmonella* serovars. This wide distribution of *Salmonella* on farms causes the contamination of animals and products of animal origin [18-20]. Especially in developing countries, other treatment options are being investigated because it causes great economic losses in the poultry industries and re-contamination is frequently encountered, and in parallel with this, resistance to antimicrobials develops [21, 22]. As an alternative to traditional antibiotic therapy, phages can be used in foods as a decontamination agent in addition to their medical uses, due to many advantages such as lack of critical side effects, host specificity, and protection of local microbiota [23, 24].

In this study, the new lytic phage against *Salmonella spp.* was isolated from the water sample province in Ankara, morphologically characterized, determined the host range on 25 *Salmonella spp.* and named as vB_SiM_12 phage. The lytic effect of vB_SiM_12 phage was compared with commercial phage products and determined that all showed a broad lytic activity to *Salmonella spp.* strains. Since bacteriophages are effective for their own species, it is important to find isolates with a wide host range in the phage selection. In addition, the extreme diversity within each bacterial specie influences the phage selection. For example, more than 2,500 serovars of *Salmonella* species have been reported. This also affects phage sensitivity [25, 26]. Gencay et al. (2019) determined that the combination of phage type and receptor is the main determinant of host spacing in their study of the characterization of *Salmonella* phages isolated from various sources [27]. Choi et al. (2017) reported that two *Salmonella* phages isolated in their study degraded five *Salmonella* serotypes [28]. A total of 58 *Salmonella* phages were isolated from wastewater treatment plants, riverside wastewater, lakeside farm waste, and poultry in the Huang et al. (2018) study [29]. Bao et al. (2015) isolated two lytic bacteriophages from sewage wastes of chicken farms and reported that both phages belong to the *Myoviridae* family as a result of cell morphology examination by TEM [30].

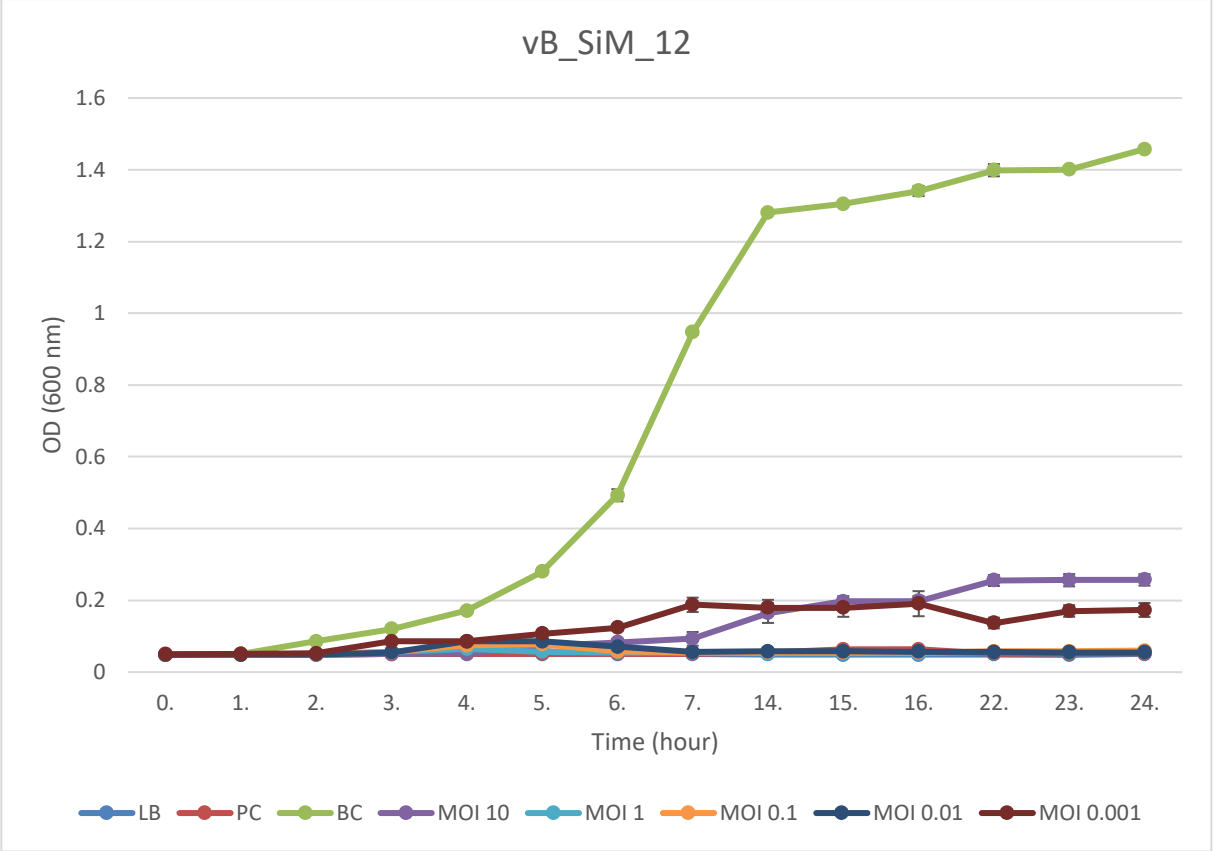

Figure 3. Bacterial growth was monitored by measuring the OD 600 at various time points. In a 96-wellplate, exponential culture was inoculated with dilutions of vB_SiM_12 phage lysate for MOIranging from 0.001, 0.01, 0.1, 1, and 10. The plates were incubated at37°C with orbital shaking for 24 hours and OD of 600 nm was measured using a Microtiter Plate Reader. As a negative control, three control samples were included: the bacterial control (BC) was inoculated with the host bacteria only and the phage control (PC) and the LB control (LB) were inoculated only. The bacterial density was recorded at 1-hour intervals over the period of 24 hours. All tests were performed in quintuplicate, and each experiment was performed duplicate. Results are presented as mean values ± SD.

-The newly isolated phage showed lytic activity on all strains. Bacterial growth with dilutions of vB_SiM_12 phage with lytic activity (MOI ranging from 0.001, 0.01, 0.1, 1, and 10) was monitored by measuring the OD 600 in a 96-well plate for 24-h. All samples being added with bacteriophages remained below OD values of the untreated controls throughout the 24-h incubation period. It is important to determine the MOI value which indicates the optimal growth rate of host bacteria and phage to ensure convenience in production and application. Ge et al. (2022) reported that Phage LP31 Salmonella phage with a wide host range achieved the highest titer at low MOI (0.01) [31]. There was no significant difference between the bacteriophage concentrations used in our study. Observation of some reduction in effect only at very high and low MOI values demonstrated that phages are effective up to a certain antimicrobial activity limit and that increasing the titer cannon affect the activity significantly even causing a decrease. Considering that high MOI values may cause increased bacterial resistance [32, 33]. The necessity of testing the dose and duration of the bacteriophage to be used beforehand and the importance of applying the phage at the appropriate dose and frequency emerge.

Leshkasheli et al. (2019) revealed that with an in vitro turbidity test that two phages significantly inhibited MDR *Acinetobacter baumannii* growth at low (0.01) and high (100) MOIs [34]. Sevilla-Navarro (2020) conducted a study with two newly isolated *Salmonella* bacteriophages and investigated the use of bacteriophages as disinfectants against the most common serovars (*S.* Infantis and *S.* Enteritidis) in farm poultry production for a week. The most bacterial reduction was obtained at the end of the second application at phage concentrations of 10^8 and 10^3 PFU/mL for *S.* Infantis and *S.* Enteritidis, respectively ($p < 0.05$). It has been reported that the lowest bacterial concentration in both serovars was observed at the end of the 5th day of phage application [35]. The results of this study are promising in terms of demonstrating the potential for use of phages as disinfectants.

Imklin et al. (2020) conducted a study with two isolated novel *Salmonella* phages from drainage water and tested their antibacterial activity on scouring pads and dish sponges. They observed a decrease in approximately 3 log CFU/mL bacteria because of phage application at room temperature [36]. These observations have been recognized as a useful preliminary analysis model to evaluate the phage activity as an antimicrobial agent before testing in vivo models and biocontrol applications.

4. CONCLUSION

In conclusion, we isolated a vB_SiM_12lytic bacteriophage specific to *Salmonella* spp. and found that *Myoviridae* family morphology. It had broad lytic activity and can be a product open to development in the fight against the agent in the food and livestock fields.

5. MATERIALS AND METHODS

5.1. Origin of strains

In this study, 25 *Salmonella* spp. (23 *S.* Infantis, 1 *S*. Enteritidis, 1 *S*. Typhimurium) strains were included. All bacterial strains used in this study were obtained from culture collections of the Department of Veterinary Science, Kepsut Vocational School, Balkesir University.

5.2. Bacteriophage cocktails

Two bacteriophage cocktails (INTESTI and ENKO bacteriophage) were bought from the George ELIAVA Institute Pharmacy, Georgia.

5.3. Bacteriophage isolation

The environmental water sample was taken in the Cubuk River (39.965131, 32.858678), Ankara. It was then quickly transported to the laboratory and stored in refrigerator at +4 ºC until use. Bacteriophage isolation was performed by the phage enrichment technique of Kropinski et al. (2009) [37]. The water sample was centrifuged at 10000 rpm and filtered. *Salmonella* spp. 12 strain (host bacteria) culture was cultivated in x2 Luria Bertani (LB) broth medium enriched with $CaCl_2$ and $MgSO_4$ and left for one-night incubation at 37 °C. After incubation, the suspension was centrifuged at 10000 rpm for 10 minutes, cleansed of the cell debris and particles.

The spot test method was applied to determine phage presence. By planting strips from fresh bacterial culture, 10 µl of each was dropped from the phage suspension to the cultivated areas. After one night, the zones in these areas were detected [38].

The phage content was enriched to increase the titer of the phage suspension to be used. In order to enrich the phage, the phage was combined with the host bacteria at appropriate dilution and double layer agar (DLA) method was applied to this mixture. At the end of the incubation period, the top agar layer was removed and filtered by centrifugation. The phage titer of the filtrate was determined as 'plaque forming unit' (PFU) [39, 40].

The formed plaques were cut from the areas where they were found with a sterile pasteur pipette and transferred to LB broth medium for single plaque isolation. Fresh bacterial culture was added to it and then incubated at 37 °C overnight. The next day, the phage suspension was centrifuged and filtered. Phage plaques were demonstrated using the DLA method. For samples that did not show a single type of plaque on the Petri dish, the process was continued until a uniform type was seen [41, 42].

To obtain the concentrated phage, DLA was applied to the dilution where the phage and host achieved the most appropriate growth together. The next day, the soft agar portion was taken with a Dragalski spatula and filtered. The phage titer was determined as 'plaque forming unit' (PFU) using the DLA method.

The phage, which was isolated and purified as a single type, was precipitated with PEG 8000/NaCl. 7.5 mL of 20% PEG 8000/2.5 NaCl was added to 30 mL of phage stock and incubated on ice for 1 h. Then it was centrifuged at 12000 rpm for 15 min. Then the pellet was resuspended in 1 mL of TE (1 M Tris (pH 8), 0.5 M EDTA (pH 8), 5 M NaCl) solution and on ice with the addition of 200 µL of 20% PEG 8000/2.5 NaCl for 1 h. The supernatant was discarded by centrifugation at 14000 rpm for 10 min and the pellet was suspended with 200 µL of TE. This suspension was used in phage morphology studies.

5.4. Morphological analysis

The prepared phage suspension was spread on a carbon-coated grid, waited for 1 min, and stained negatively by adding 0.5% uranyl-acetate [43]. The head and tail structure (if any) lengths of the phages were measured with a TEM microscope (Hitachi HT7800) and their classification was made in accordance with the International Viral Taxonomy (ICTV) guidelines.

5.5. In vitro susceptibilities of bacteriophages

The efficacy of lytic phage and commercially prepared phage cocktails were evaluated in a spot test. Briefly, the overnight culture of *Salmonella* spp. isolates was streaked on LB agar. A novel isolated phage titer was adjusted to 10^8 PFU/mL). The commercial phage preparations were used directly. Because the exact concentrations were not estimated.

5.6. In vitro turbidity assays

The in vitro lytic efficiency of the phage was examined at several MOIs (multiplicity of infection). Bacterial density was measured over time to determine the course of phage infection. A liquid culture of bacteria was incubated with LB. Bacterial concentrations were adjusted to 10^6 CFU/mL. 20 µL of bacterial suspension (10^6 CFU/mL), 20 µL of phage suspension at different concentrations, and 160 µL of LB broth were added to the 96-well microplate. Phage lysate was diluted to 10^4, 10^5, 10^6, and 10^7 PFU/mL, corresponding to MOIs of 0.01, 0.1, 1, and 10, respectively. Each phage-host combination at specific MOIs was performed in quintuplicate, and each experiment was performed in duplicate. Bacterial growth was monitored by measuring the OD 600 at various time points. The microtiter plate was placed in a microplate reader set at 37 °C and the first measurement at OD 600 nm was taken immediately. Controls for plate sterility, phage suspension sterility, and bacterial growth without phage addition were also included. The plates were incubated at 37°C with orbital shaking for 24-h and OD at 600 nm was measured using a Microtiter Plate Reader (Thermo Multiskan FC) at 60-minute intervals. The microplate was sacked for 5 s before each measurement.

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