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Effects of abiotic factors on the stability and infectivity of polyvalent coliphage

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ABSTRACT

Bacteriophage has attracted growing interest as a promising therapeutic agent for pathogenic bacteria, especially for antibiotic-resistant bacteria. However, the various abiotic conditions could impact the stability of phages and further threat host–virus interactions. Here, we investigated the stability and lytic activity of virulent polyvalent coliphage (named PE1) by double-layer plaque assay. PE1 can efficiently infect both the drug-sensitive *Escherichia coli* K12 and multidrug-resistant *E. coli* NDM-1 even after prolonged storage at 4 °C for up to two months. Results showed that PE1 exhibits an outstanding stability to infect *E. coli* strains under a wide range of thermal (4 °C–60 °C) and pH (4–11) conditions, which covers the thermal and pH variations of most wastewater treatment plants. Moreover, PE1 exhibited high resistibility to heavy metals exposure including Cu^{2+}, Cd^{2+}, Co^{2+}, and Cr^{3+} at the concentrations below 0.5 mM, and an excellent resistant ability to the variation of ionic strength, which still retained strong infectious ability even treated with saturated sodium chloride solution (350 g/L). This work shows that polyvalent phage PE1 has a strong adaptive capacity to various abiotic factors and should be a good candidate of being an antibacterial agent, especially for antibiotic-resistant bacteria control in sewage.

Key words: abiotic factor, antibiotic resistant bacteria, *Escherichia coli*, phage stability, polyvalent bacteriophage

HIGHLIGHTS

- A virulent polyvalent coliphage named PE1 can propagate fast and effectively in both drug sensitive and drug resistant bacteria even after it has been stored for two months.
- PE1 exhibited a strong resistant ability to the variations of common environmental factors including thermal, pH, ionic strength and several heavy metals, which could be a good candidate to be used as the antibacterial agent.

INTRODUCTION

With the growth of urban population and the development of industrial production, water shortage becomes increasingly serious (Han *et al.* 2018). Recycling, reuse and reclamation of municipal wastewater have been considered as viable solutions to meet the growing water demand (Liu & Persson 2013; Zhu & Dou 2018). However, the quality of reused water may be impaired by pathogen contamination, particularly antibiotic resistant bacteria residues, which pose a direct threat to human health and ecological security (Meric & Fatta Kassinos 2009). Therefore, there is a critical need for suitable antimicrobial technologies to prevent and control waterborne diseases.

Nowadays, many treatment technologies can effectively remove most pathogenic bacteria from the wastewater, but they still have many disadvantages. Although ultraviolet disinfection is the preferred technology for inactivation of pathogens in wastewater, it still has many disadvantages. The high energy consumption (Miklos *et al.* 2018) and limited sterilization ability of ultraviolet disinfection which could cause health and safety concerns associated with the irradiated effluent (Guo & Kong 2019) have caused the manufacturers to seek out much safer ways to disinfect the effluents from sewage treatment plants. Liquid chlorine disinfection also has some inevitable problems, such as security risk of storing liquid chlorine and carcinogenic risk of disinfection by-products (Richardson *et al.* 2007; Villanueva *et al.* 2015). Compared with these common used treatment technologies, bacteriophage (phage) has attracted a growing interest due to its unique advantages. For example, it can exclusively infect bacteria and replicate within bacterial host without any residuals, making them harmless to humans and safe to use for wastewater treatment (O'Flaherty *et al.* 2009; Worley-Morse & Gunsch 2015).

Furthermore, phage is widely distributed in the natural environment with a total population of about 1×10^{31} on earth (Ackermann 2001), providing a rich antibacterial resource pool for pathogen control (Rohwer *et al.* 2009).

However, the lytic activity and stability of phage may be influenced by many factors, such as bacterial growth stage, calcium and magnesium concentrations. Bacteria in logarithmic growth phase were more susceptible to phage (Ibarra *et al.* 2010), and a certain concentration of calcium and magnesium ions may increase the phage infectivity (Lu *et al.* 2003; Ul Haq *et al.* 2012). Other environmental conditions like temperature, pH and salt concentration have also been investigated in several studies. It was reported that lytic activity of phage vB_SflS-ISF001 against multidrug-resistance strain in contaminated foods maintained well after being treated with different concentrations of saline, and remained at high levels after being incubated at −20 °C to 50 °C for 1 h but decreased significantly at 60 °C (Shahin & Bouzari 2018), while another study reported that phages against bacterial canker in kiwi exhibited good tolerance to the high temperature condition at 60 °C (Yin *et al.* 2018). When dealing with the food-borne pathogenic bacteria at 70 °C, the antibacterial activity of phage SFP10 was reduced by 54% and was entirely inactivated at 75 °C, while it showed a high degree of stability between pH 4 and 10 (Park *et al.* 2012). Therefore, phage having different hosts or a particular application environment could show different adaptive capacities to environmental factors.

In this study, we investigated the stability and antibacterial activity of virulent coliphage (named PE1), which was isolated and purified from the sewage treatment plant using *Escherichia coli* (*E. coli*) K12 as the host. The effect of common abiotic factors, including temperature, pH, salinity and heavy metals, on the lytic activity of phage PE1 were determined with drug-sensitive (*E. coli* K12) and drug-resistant bacteria (*E. coli* NDM-1, which is resistant to β-lactam antibiotics carrying the plasmid-encoded $bla_{\text{NDM-1}}$ gene) (Bonomo 2011) as the host.

MATERIALS AND METHODS

Bacterial strains and cultural conditions

E. coli K12 (ATCC 10798) was used as the host for phage isolation and propagation, and other six bacterial strains listed in Table 1 were used for host range test. A single colony of each strain was cultured in tryptic soy broth (TSB) medium at 30 °C overnight with shaking at 150 rpm. The double-layer method was performed with a base agar (tryptone base layer agar, TBA) and a soft agar (tryptone soft agar, TSA). The isolated phages were stored in SM buffer (50 mM Tris-HCl [pH 7.5], 8 mM $MgSO_4$, 0.1 mM NaCl, 0.01% gelatin) with a few drops of chloroform at 4 °C (Li *et al.* 2017).

Bacteriophage isolation and purification

Wastewater sample (10 mL) was collected from the oxidation ditch from the Jinhai municipal wastewater treatment plant in Chengdu, China, by means of grab sampling. Then 100 mL TSB was added into the wastewater sample for overnight incubation at 30 °C with shaking at 150 rpm. The phage stocks were isolated as previously described with some modifications (Di Lallo *et al.* 2014). Briefly, phages were harvested from the medium using sodium pyrophosphate method and further filtered through 0.22-μm polyamide membrane to remove larger particles. Then phages in filtrate were precipitated by polyethylene glycol 8,000 and resuspended in SM buffer as the phage stock.

With double-layer method, phage PE1 was further enriched and purified from phage stock using *E. coli* K12 as the host. A single phage plaque from the lawn of *E. coli* was harvested and diluted in SM buffer, and then the obtained phage solution

Table 1 | Host range of polyvalent phage PE1

Bacteria	ATCC #	Family	Infectivity[a]
E. coli K-12	10,798	Enterobacteriaceae	++
E. coli NDM-1	BAA-2452	Enterobacteriaceae	++
Shewanella putrefaciens CN32	BAA-453	Shewanellaceae	–
Shewanella oneidensis MR-1	700,550	Shewanellaceae	–
Pseduomonas putida F1	700,007	Pseudomonadaceae	–
Pseudomonas putida MnB1	23,483	Pseudomonadaceae	–
Pseudomonas putida	12,633	Pseudomonadaceae	–

[a]Spot tests and plaque assay showed infection (++) or no infection (–).

was further purified at least three times by repeating the same process above to remove the contaminated ones. Finally, the solution of phage PE1 was centrifuged at 8,000 × g for 10 min and filtered through 0.22-μm polyamide membrane. The filtrate was stored at 4 °C with a few drops of chloroform for further use, and the influence of storage time on the activity of phage PE1 was evaluated at different storage time points (day 0, 15, 30, and 60) by determining plaque formation capability.

Bacteriophage host range and bacterial challenge tests

The phage host range was determined by the spot test assay on the potential host lawn. 5 μL phage suspension (~10^9 PFU/mL) was added to the potential host lawn and incubated at 30 °C overnight. The results were further confirmed by measuring the optical density at 600 nm (OD_{600}) of liquid medium during the bacterial batch growth of host. Specifically, the bacteria at exponential phase was transferred to a 200 mL sterilized Erlenmeyer flask containing 100 mL TSB medium to a final concertation of 10^6 CFU/mL. The flasks were placed into an orbital shaker (150 rpm, THZ-92A, Shanghai Boxun Medical Biological Instrument Corp., China) at 30 °C and OD_{600} of the medium was measured at given time points. For phage-treated groups, the bacteria were infected by phage PE1 at the optical multiplicity of infection (MOI) of 1 initially both for *E. coli* K12 and NDM-1, which were pre-tested as previously described (Lu *et al.* 2003).

Transmission electron microscopy

The purified phage PE1 (~10^8 PFU/mL) was loaded onto the carbon-coated copper grids and then negatively stained with 3% phosphate tungsten acid for 5 min. The stained specimens were air dried and observed with a JEOL 1230 transmission electron microscope at 120 kV. Based on the morphology, phage identification and classification were conducted according to the report of International Committee on the Taxonomy of Viruses (King *et al.* 2012).

Bacteriophage latent time, burst time and burst size

One-step growth curves were conducted to determine the latent time, burst time and burst size of phage PE1 as previously described with some modifications (Kropinski *et al.* 2009). Briefly, phage PE1 was added at optimal MOI into 1 mL TSB medium containing different host bacteria at the mid-exponential phase ($OD_{600} = 0.1$, about 1×10^8 CFU/mL) and allowed to adsorb at 30 °C for 5 min. Then the mixture was centrifuged at 12,000 × g for 2 min to remove the free phages, and the sediment was resuspended with the same volume of medium. 100 μL of the resuspended culture was added into 50 mL TSB medium for incubating at 30 °C with shaking at 150 rpm. Samples (1 mL) were taken at 10 min intervals and immediately tested by double-layer plaque assay for phage titer. Assays were conducted in triplicates, and the plaque counts were used to generate the one-step growth curve. The latent time, burst time, and burst size were calculated from the one-step growth curve.

Effects of different abiotic factors on bacteriophage activity

The phage titer of acquired filtrate used for impact assessment of abiotic factors was determined at optimal MOI by double-layer plaque assay at 30 °C in triplicates. Four major abiotic factors, including temperature, pH, ionic strength, and heavy metals, were chosen to investigate their effects on the activity of phage PE1. For each factor, phage PE1 was treated for 1 h according to the method described previously (Ul Haq *et al.* 2012). Then the phage solution was serially diluted 10-fold with SM buffer and further mixed with different host bacteria at the exponential phase in 1 mL TSB medium containing 10 mM $MgSO_4$. The mixture was immediately added into 6 mL of un-solidified TSA that was kept in water bath at 46 °C and poured onto the surface of TBA plate. After incubation at 30 °C overnight, the number of plaques on each plate was recorded and calculated as phage titer.

Thermal and pH stability of phage PE1

To evaluate the thermal stability of the phage PE1, 1.0 mL of phage solution was treated at a series of temperatures (4, 20, 30, 40, 50, 60, 70 and 80 °C) using refrigerator or electric water bath for 1 h. The viability of phage was tested as phage titer for different host bacteria. Each trial was carried out in triplicates. The pH stability test for phage PE1 was carried out as previously described (Choudhury *et al.* 2019). 1.0 mL of phage solution was treated under specific pH condition at 30 °C for 1 h in triplicates, then the phage titer of each treated sample was tested by double-layer method as mentioned above. The solution was modified with citrate-sodium citrate buffer, phosphate buffer, borax-boric acid buffer, and carbonate buffer separately to achieve pH values 4.0–5.0, 6.0–7.0, 8.0–9.0, and 10.0–11.0, respectively.

Based on the results of single factor experiments above, response surface methodology (RSM) with central composite design (CCD) was further used to evaluate the co-effects of temperature (20–60 °C) and pH (4.0–11.0) on phage activity.

Effects of ionic strength and heavy metals on phage activity

To assess bacteriophage stability at various salinities, different concentrations of NaCl in SM buffer (pH 7.5) were prepared with final salinity of 10, 50, 200 and 350 g/L, respectively (Choudhury *et al.* 2019). Phage PE1 was treated by different salinities at 30 °C for 1 h in triplicates, followed by phage titer determination. To study the effects of heavy metal ions on the phage activity, a series of $CuCl_2$ solution (0.005, 0.05, 0.5, and 5 mM) and four different heavy metals solutions, i.e., $CrCl_3$, $CdCl_2$, $CoCl_2$ and $CuCl_2$, at a final concentration of 0.5 mM were assayed. For each condition, phage PE1 were treated by the filter sterilized heavy metal solution for 1 h in triplicates and tested against host bacteria in double-layer plaque assay to check the viability of phages.

Statistical analysis

For RSM-CCD experiments, the design, regression and graphical analysis of the data was conducted with Design-Expert 8.0.6.1. According to 2 variables with 5 levels, 13 experiments were designed in CCD model. Analysis of variance (ANOVA) were applied to assess the validity and adequacy of the model and to investigate the probable interactions between the effects of pH and temperature on phage stability.

All tests were conducted in triplicates and results were expressed as mean ± standard error. Using Origin 2018b, the results were analyzed by ANOVA after normality analysis with Kolmogorov-Smirnov test and homoscedasticity analysis with Levene's test, and Student's *t* test was used to compared two conditions ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

RESULTS AND DISCUSSION

Polyvalent coliphage isolation and characterization

Virulent phage PE1 was isolated and purified from the oxidation ditch of Jinhai municipal wastewater treatment plant in Chengdu by successive single plaque isolation. PE1 was able to efficiently and stably infect *E. coli* K12, forming clear and large plaques (Figure 1(b)). Host range tests showed that phage PE1 was highly specific to inactivate *E. coli* stains without infecting other five strains belonging to Shewanellaceae or Pseudomonadaceae families (Table 1). Besides *E. coli* K12, Coliphage PE1 could also significantly suppress the growth of *β*-lactam-resistant *E. coli* NDM-1 (Figure 2(a)). The long contractile tail and regular polyhedral head observed under TEM (Figure 1(a)) indicated phage PE1 belongs to *Myoviridae* (Iwasaki *et al.*

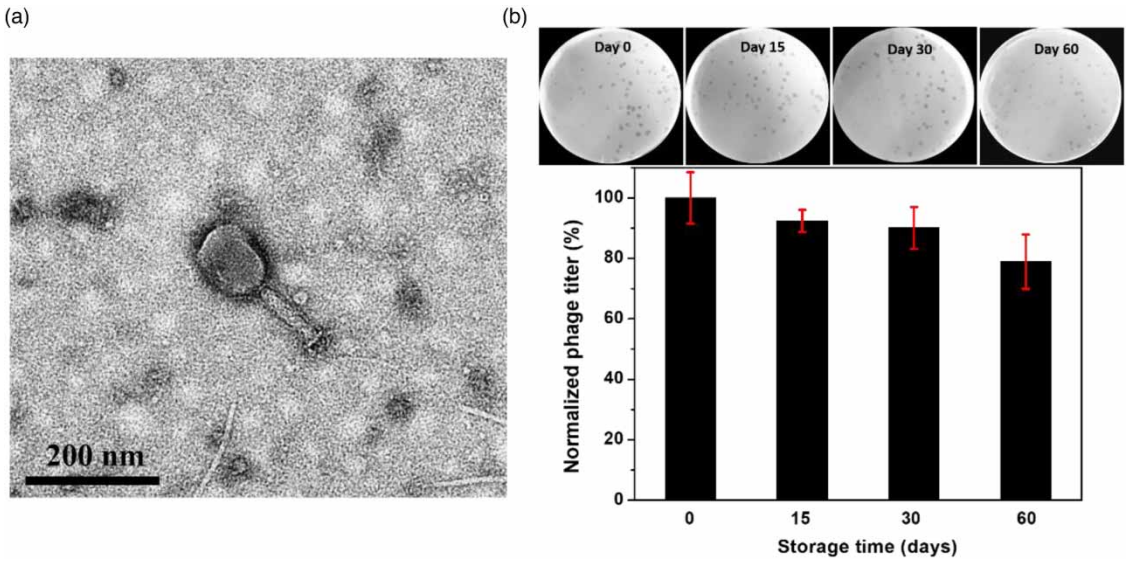

Figure 1 | Morphology of PE1 (a) and influences of storage time on its plaque formation capabilities (b). Plaque forming tests of phage PE1 in *E. coli* K12 at different preservation time points were demonstrated and the histograms show the fraction of the remaining phage activity. The phage titer of the fresh one (Day 0) was defined as 100%. These different storage times showed no significant effect on the phage activity ($p > 0.05$).

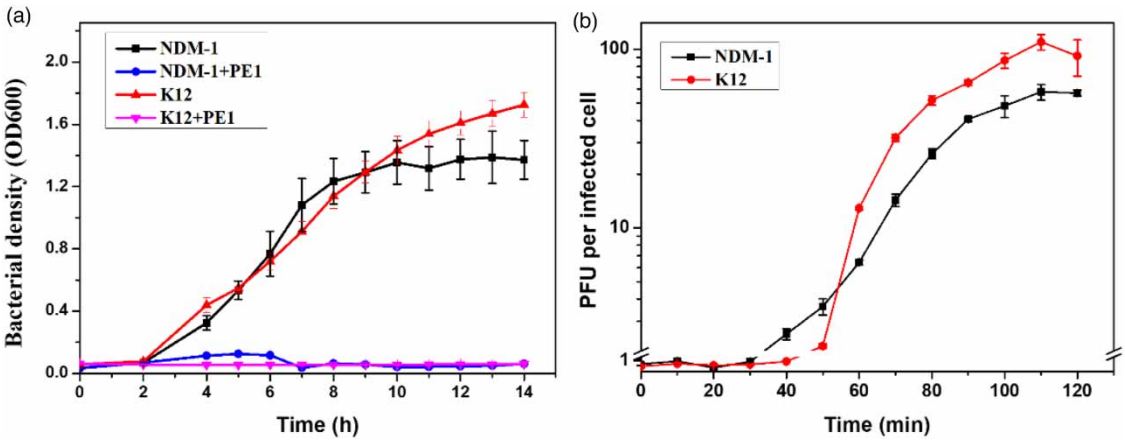

Figure 2 | Polyvalent phage PE1 infected both *E. coli* K12 and NDM-1. The growth curves of each bacterium inoculated with polyvalent phage PE1 at MOI of 1 (a) and the relative one-step growth curve of phage PE1 (b).

2018). The one-step growth curve of PE1 (Figure 2(b)) showed that when *E. coli* NDM-1 and *E. coli* K12 was the host separately, the phage latent time was 30 and 40 min, respectively, while the correspond burst size was 57 ± 5 and 86 ± 8 PFU/cell, respectively. These results are similar to the reported values for coliphage and also consistent with previously observed results showing that latent time and burst size were positively correlated (Yu *et al.* 2015; Yu *et al.* 2017). These growth parameters indicate phage PE1 can propagate fast and effectively in both drug-sensitive and drug-resistant bacteria, suggesting its potential application as an antibacterial agent.

The acquired phage solution with high titer (10.2 ± 0.01 $\log_{10}$ PFU/mL with *E. coli* K12 as host) was stored at 4 °C for subsequent uses. To enable stable phage activity throughout this study, the influence of storage time on the activity of phage PE1 was evaluated. Phage PE1 still retained strong infectious ability after being stored for two months (Figure 1(b)), and it showed similar capability of plaque-forming on the 15th day (92.4 ± 3.6%) and 30th day (90.0 ± 6.9%) compared with the fresh one (100 ± 8.4%). Even after two months, the antibacterial activity of phage PE1 only slightly decreased to 78.9 ± 8.9% with no significant difference with others, improving its potential for practical applications.

Thermal and acid-base stability of phage PE1

The lytic activity and stability of phage can be influenced by different physicochemical parameters. In this study, we examined the influences of temperature, pH, ionic strength and heavy metals, which are important parameters for applying phage technology to wastewater treatment.

The thermal stability of phage PE1 was demonstrated under different temperatures (Figure 3(a) and 3(b)). The lytic activity of PE1 was slightly reduced after incubated at 50 °C, but still kept strong infectious ability (9.9 ± 0.02 $\log_{10}$ PFU/mL with *E. coli* K12 as host, Figure 3(a)) compared with the group that was incubated at 4 °C (10.2 ± 0.01 $\log_{10}$ PFU/mL). Phage PE1 kept stable under temperatures ranging from 4 °C to 50 °C, which was similar to the previous reported thermal stability of coliphages (Li *et al.* 2010; Xu *et al.* 2018). At 60 °C, the phage titer only reduced one order of magnitude with *E. coli* K12 as the host and less than one order of magnitude with NDM-1 as the host (Figure 3(b)), indicating better lytic activity than phage vB_EcoS-B2 infecting multidrug-resistant *E. coli* (Xu *et al.* 2018). However, when the temperature exceeded 70 °C, phage PE1 lost its activity completely. These results indicate that phage PE1 has a high stability over a wide range of temperatures, which is capable of adjusting to the changing temperature in most of wastewater treatment plants (Caicedo *et al.* 2019).

The effect of pH on phage PE1 was characterized in the range of 4.0 to 11.0 (Figure 3(c) and 3(d)). PE1 was able to maintain its lytic activity (varied from 9.8 ± 0.06 to 10.2 ± 0.02 $\log_{10}$ PFU/mL with *E. coli* K12 as the host, Figure 3(c)) after 1 h incubation in a pH range of 6.0–9.0, and showed the best lytic capacity at pH 7.0. These results indicated that phage PE1 could maintain high lytic activity under mild alkaline/acidic or neutral conditions, which is in agreement with previous studies (Li *et al.* 2010; Shahin & Bouzari 2018; Ding *et al.* 2020). Whereas, extreme pH environment might pose hindrance to phage stability, the remaining phage titer of PE1 decreased more than three orders of magnitude at pH 4.0, 5.0, 10.0 and 11.0 (Figure 3(c)), and acidic conditions had a greater impact than alkaline environment. Even so, PE1 also showed a greater resistance to acidic conditions than previously reported engineered *E. coli* phage EEP, which could be barely detected

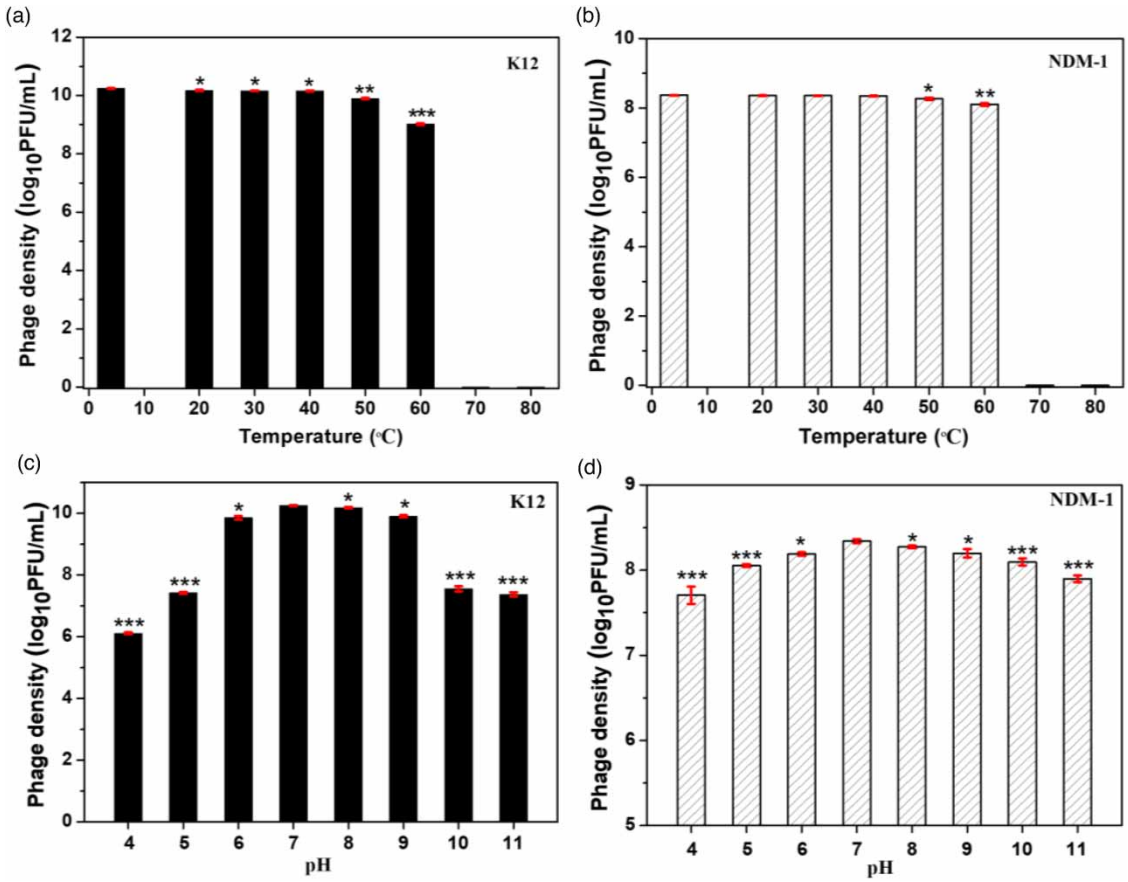

Figure 3 | Thermal and acid-base stability of phage PE1. Plaque forming tests of phage PE1 with *E. coli* K12 (a, c) and NDM-1 (b, d) as the host after treated with different temperature or pH conditions were demonstrated. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with the group at 4 °C or under pH = 7.0.

(<10 PFU/mL) (Li *et al.* 2010) while PE1 was able to maintain its infectivity (6.1 ± 0.02 $\log_{10}$ PFU/mL) after incubation at pH 4.0 for 60 min. Interestingly, when infecting multidrug-resistant *E. coli* NDM-1 (Figure 3(d)), the lytic capacity of polyvalent phage PE1 was less affected compared with the situation to infect the drug-sensitive *E. coli* K12, and demonstrated better adaptive capacity to alkaline environment (Figure 4(b)).

The interactive effects on phage activity between pH and temperature were further investigated through response surface models. With nonlinear regression method, the following equations in terms of actual values of pH (X_1) and temperature (X_2) was obtained by modeling the experimental results with *E. coli* K12 (Equation (1)) and *E. coli* NDM-1 (Equation (2)) as the host, respectively:

$$Y = 9.17 - 0.82 * X_2 - 4.59 * X_1^2 - 0.55 * X_2^2 - 1.14 * X_1^2 * X_2 + 0.35 * X_1 * X_2^2 + 2.32 * X_1^2 * X_2^2$$
$$R^2 = 0.99 \quad (1)$$

$$Y = 8.63 + 1.34 * X_1 - 1.28 * X_2 - 0.24 * X_1 * X_2 - 0.87 * X_2^2 - 0.50 * X_1^2 * X_2 - 1.49 * X_1^3 - 1.58 * X_1^4$$
$$R^2 = 0.99 \quad (2)$$

The high values of coefficient of determination ($R^2 = 0.99$ for *E. coli* K12, $R^2 = 0.99$ for *E. coli* NDM-1) and low *p*-values (<0.0001 for *E. coli* K12, <0.0001 for *E. coli* NDM-1, Table 2) implied that these two models were significant, and the *p*-values of lack of fit in both models were greater than 0.05 (not significant) indicating that these two models fit the data well. For *E. coli* K12, the contour plots (Figure 4) and ANOVA analysis results showed that X_2, X_1^2, X_2^2, $X_1^2X_2$, $X_1X_2^2$ and $X_1^2X_2^2$ were the significant terms with low *p*-values (<0.05, Table 2), while for *E. coli* NDM-1, X_1, X_2, X_1X_2, X_2^2, $X_1^2X_2$, X_1^3

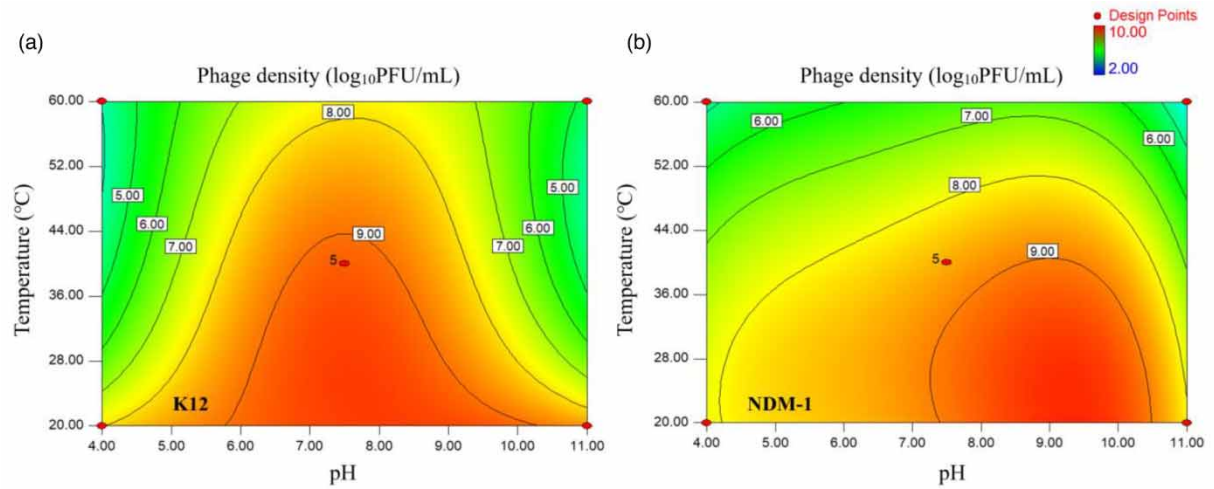

Figure 4 | 2D contour plots of the lytic capacity of PE1 related to the interactive effects of pH and temperature.

Table 2 | Data of ANOVA analysis obtained by response surface model

Host	Source	Sum of Squares	df	Mean Square	F Value	*p*-value Prob> F	
E. coli K-12	Model	143.13	6	23.86	2,091.83	<0.0001	Significant
	X_2-Temperature	2.69	1	2.69	235.98	<0.0001	Significant
	X_1^2-pH^2	120.13	1	120.13	10,533.61	<0.0001	Significant
	X_2^2	1.70	1	1.70	148.83	<0.0001	Significant
	$X_1^2X_2$	2.61	1	2.61	228.82	<0.0001	Significant
	$X_1X_2^2$	0.48	1	0.48	42.36	0.0006	Significant
	$X_1^2X_2^2$	10.74	1	10.74	941.90	<0.0001	Significant
	Residual	0.068	6	0.011			
	Lack of Fit	0.038	2	0.019	2.5	0.1974	Not significant
	Pure Error	0.030	4	0.0008			
	Cor Total	143.20	12				
E. coli NDM-1	Model	88.23	7	12.60	1,823.01	<0.0001	Significant
	X_1-pH	1.45	1	1.45	209.27	<0.0001	Significant
	X_2-Temperature	6.52	1	6.52	942.42	<0.0001	Significant
	X_1X_2	0.24	1	0.24	34.02	0.0021	Significant
	X_2^2	4.99	1	4.99	720.99	<0.0001	Significant
	$X_1^2X_2$	0.50	1	0.50	72.65	0.0004	Significant
	X_1^3	4.42	1	4.42	639.93	<0.0001	Significant
	X_1^4	58.05	1	58.05	8,395.09	<0.0001	Significant
	Residual	0.035	5	0.0007			
	Lack of fit	0.0008	1	0.0008	1.14	0.3450	Not significant
	Pure error	0.027	4	0.0007			
	Cor total	88.27	12				

and X_1^4 were the significant terms with low *p*-values (<0.05, Table 2). In both cases, compare with temperature, pH condition showed more significant effect on the stability of phage.

Effect of ionic strength on phage stability

To achieve large-scale production of phage suspensions with high titer for practical applications, ion exchange chromatography is usually used for phage purification (Jończyk-Matysiak *et al.* 2019). In this method, the repulsion forces between charged molecules can be neutralized with the increase of salt concentration during sample loading (Yuan *et al.* 2000), which makes denser molecules absorbed on the chromatographic surface. Therefore, determination of phage stability

under different ionic strengths is very important. Here, PE1 was treated by a series of NaCl solutions for 1 h with final salinity of 10, 50, 200 and 350 g/L, respectively.

With the increase of ionic strength (Figure 5), the lytic activity of PE1 decreased but still retained a strong ability to infect *E. coli* K12 ($9.3 \pm 0.04 \log_{10}$ PFU/mL) and *E. coli* NDM-1 ($5.7 \pm 0.04 \log_{10}$ PFU/mL), even treated with saturated sodium chloride solution (350 g/L). The result is similar to phage vB_SflS-ISF001 against multidrug-resistant *Shigella* spp. in contaminated foods, which is able to tolerate different concentrations of saline ranging from 1 to 35% (Shahin & Bouzari 2018). Compared with phage T4 using *E. coli* DSMZ 613 as the host, which was reported to be stable in NaCl solution with concentrations ranging from 0.1 to 1.5 M, but the infectivity was reduced at 2 M (Smrekar *et al.* 2008), phage PE1 showed a stronger resistant ability to sodium ions, indicating ionic strength should not be the limiting factor for phage purification and application.

Effects of heavy metals on phage stability

Divalent metal ions are a crucial factor influencing phage activity (Jończyk-Matysiak *et al.* 2019), such as Ca^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+}, and Fe^{2+}, which usually play a prompting role for phage adsorption, binding lytic enzymes and sustaining phage activity (Delbrück 1948). It was demonstrated that compared with phage-only treatment, another 2-logs reduction in pathotype extraintestinal pathogenic *E. coli* levels in human blood was archived when adding a phage cocktail containing 5 mM Ca^{2+}, Mg^{2+} or Fe^{2+} (Ma *et al.* 2018), whereas a phage cocktail containing 5 mM Zn^{2+}, Co^{2+} or Cd^{2+} depressed the stability of phage BVPaP-3 when using *Pseudomonas aeruginosa* as the host (Ahiwale & Kapadnis 2016). Therefore, the phage species and the content of different divalent metals would exhibit different results. To identify the effect of divalent metal ions especially the common toxic heavy metals in wastewater treatment processes, we examined the lytic activity of phage PE1 after four different heavy metal ions treatment, including Cu^{2+}, Cd^{2+}, Co^{2+}, and Cr^{3+}, which have been proven to be less toxic to ecosystem as the reduction product of hexavalent chromium in wastewater (Jobby *et al.* 2018).

The stability of phage PE1 to infect *E. coli* K12 was undermined by all four heavy metal ions at 0.5 mM (Figure 6(a)), and the inhibitory effects on PE1 followed the order of $Cr^{3+} > Cd^{2+} = Co^{2+} > Cu^{2+}$. During these four tested heavy metal ions, Cu^{2+} exhibited the weakest inhibition effect that reduced phage titer to $9.2 \pm 0.04 \log_{10}$ PFU/mL while the lytic capacity of PE1 treated by other three metal ions decreased more than three orders of magnitude (Figure 6(a)). The phage titer of PE1 was lowest after Cr^{3+} treatment ($5.2 \pm 0.12 \log_{10}$ PFU/mL), which was five orders of magnitude lower than the control group, indicating that PE1 is very sensitive to the toxic effect of Cr^{3+}. These findings were inconsistent with the previous results that the stability of phage would be adversely affected by metals at a high concentration (Bouzari *et al.* 2008). When multidrug-resistant *E. coli* NDM-1 as the host, the inhibition effects on phage PE1 were much weaker than *E. coli* K12 as the host (Figure 6(a) and 6(b)). Cd^{2+} showed the strongest inhibition effect on PE1 to lyse host bacteria but with less than one order of magnitude reduced, while 0.5 mM Cu^{2+} barely repressed the lytic capacity of PE1 for *E. coli*

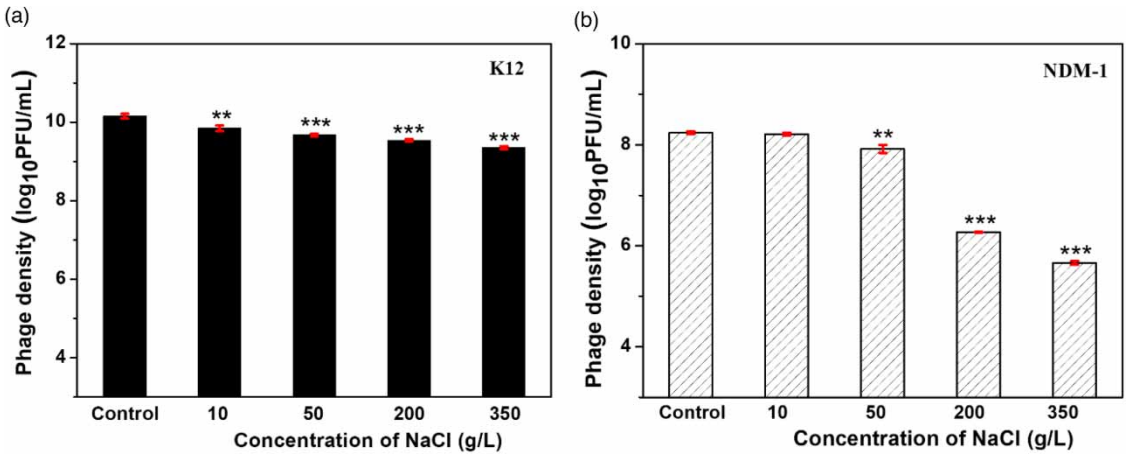

Figure 5 | Influence of ionic strength on the lytic capacity of PE1. Plaque forming tests of phage PE1 with *E. coli* K12 (a) and NDM-1 (b) as the host after treated in NaCl solutions with different concentrations were demonstrated. $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the control group.

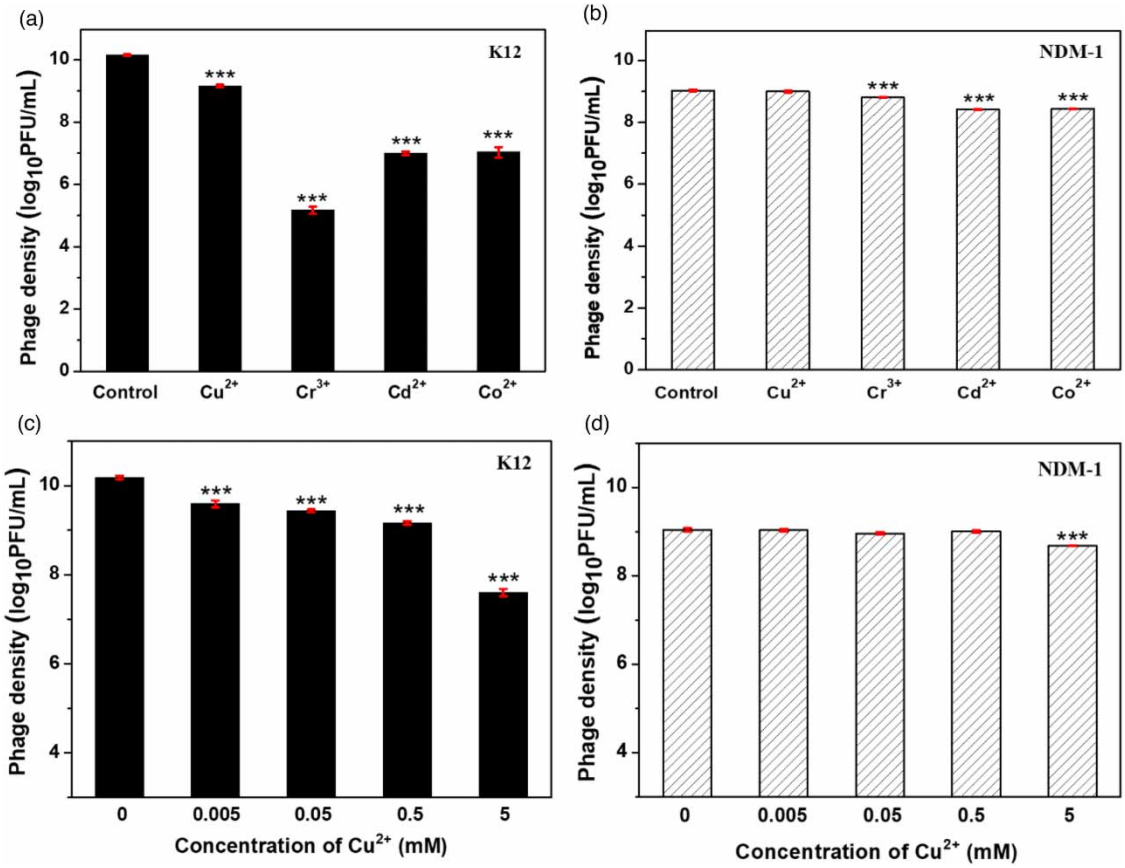

Figure 6 | Influence of heavy metals on the lytic capacity of PE1. Plaque forming tests of phage PE1 with *E. coli* K12 (a and c) and NDM-1 (b and d) as the host after treated by several heavy metal ions and different concentrations of Cu^{2+} were demonstrated. ***$p < 0.001$ compared with the control group.

NDM-1 with no significant difference compared with the control group (Figure 6(b)), indicating that when infecting different host bacteria, heavy metals exposure might have different effects on the inactivation ability of same phage.

Previous studies suggested that the infectivity property of phages could be stimulated slightly or be barely effected when exposed to various metal ions at low concentrations, including some common toxic heavy metals (Ahiwale & Kapadnis 2016; Jończyk-Matysiak *et al.* 2019). Thus, we further examined the influence of different concentrations of Cu^{2+} on the stability of phage PE1. The results showed that with Cu^{2+} concentration increased, the plaque formation capability of PE1 was decreased (Figure 6(c) and 6(d)), but the phage titers decreased less than one order of magnitude at the concentrations below 0.5 mM compared with the control group, indicating that PE1 could maintain its stability well at the low concentrations of Cu^{2+}. All the results above indicate that coliphage PE1 can keep relatively high stability after exposure to low concentration of heavy metals below 0.5 mM for one hour. But unlike the common divalent cations (Ca^{2+}, Fe^{2+} and Mg^{2+}) applied in previous reports, who can significantly increase the phage activity against host bacteria (Ma *et al.* 2018) through stimulating the adsorption of phage to host cell, the intracellular synthesis of phage progeny and the maintenance of the structure of phage proteins, the heavy metals investigated here did not show any enhancement (Figure 6), which may be related to the chelation between metal ions and phage particles (Bonnain *et al.* 2016).

The above results indicate that polyvalent phage PE1 has a strong adaptive capacity to various abiotic factors, which should be a good candidate of being an antibacterial agent applied in sewage treatment, especially for inactivation of *E. coli* NDM-1, a multi-drug-resistant super bacteria (Bonomo 2011). However, it should be noted that, the matrix is much more complex in municipal wastewater than that was analyzed here, and more detailed studies should be conducted in the future to evaluate the stability of virulent phage in practical wastewater. Furthermore, the host range test showed that phage PE1 did not infect other bacterial families included in this study (Table 1), which may decay quickly when added into the wastewater treatment

plant. Hence, more virulent phages for different hosts need to be isolated in the future, which can offset the relatively fast phage decay effectively and raise its potential for practical applications.

CONCLUSIONS

Characterization of polyvalent phage PE1 showed that PE1 was very efficient in lysing drug-sensitive *E. coli* K12 and drug-resistant *E. coli* NDM-1. Combined with its outstanding thermal and pH stability and some resistibility to common heavy metals, phage PE1 could be a good candidate to be used as an antibacterial agent, especially for multi-drug-resistant bacteria control in sewage. In addition, phage PE1 showed an excellent resistant ability to sodium ions indicating that ionic strength should not be the limiting factor for practical application, which can be purified by ion exchange chromatography to achieve large-scale production of phage suspensions with high titer. However, even under the same abiotic condition, polyvalent phage PE1 exhibited different lytic capacity when dealing with different host bacteria, which should be related with the specific infection mechanisms and deserves further investigation. This work would be beneficial for the development and application of bacteriophage technology in wastewater treatment, and more studies should be conducted to further improve the stability and infectivity of virulent phages for practical application in municipal sewage.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing financial interest.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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