The Effect of Environmental Factors on Phage Stability and Infectivity on Their Host Bacteria: a Case Study for an *Escherichia coli* Phage (T7), a *Listeria* Phage (A511), and a *Salmonella* Phage (Felix O1)

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Abstract The effectiveness of phage biocontrol depends on the activity of bacteriophage in a given environment. In order to investigate the infectivity and the stability of bacteriophages in representative environments, three virulent phages, *Listeria* phage A511, *Salmonella* phage Felix O1, and *Escherichia coli* phage T7, were subjected to different temperatures, pHs and salt concentrations (NaCl). Phage infectivity was also determined in the presence of divalent cations (Mg^{2+} or Ca^{2+}). As a result, three phages exhibited a wide range of survival rates under various environments. Phage infectivity was directly correlated with bacterial growth under the applied conditions. One exception was Felix O1 that did not kill *Salmonella* grown in low pH (4.5). The failure was attributed to defective adsorption of Felix O1. This finding is significant as it provides an explanation for the inefficient phage biocontrol. Therefore, such information is crucial to improve phage biocontrol of pathogens.

Keywords: phage biocontrol, phage inactivation, phage infectivity

Introduction

Bacteriophage (phage) biocontrol is the application of phage for the control of harmful microorganisms in food system (1-3). Advantages include: (i) specificity, phages intrinsically contain the ability to destroy the host target bacteria. The host range could be at the species or even strain level, which is advantageous because many foods require continuous presence of non-harmful micro-organisms; (ii) efficiency, phage treatment in food could be as effective as chemical sanitizers in destroying pathogens (1) and spoilage microorganisms (4, 5); (iii) non-destructive and non-thermal method to keep the freshness of food; (iv) safety, phage itself is merely a nucleic acid molecule covered by protein capsid which might contain some lipids. One might ingest 10^{13} virus-like molecules in daily life and phage can't metabolize or replicate without the respective bacterial host. Recent human trial found no side effects from phage treatment (6). In addition, as the misuse of antibiotics in food might contribute to the spread of antibiotic resistant bacteria, introducing another agent that acts differently could reduce such risks. Obviously, recent FDA approval of using *Listeria* phages in foods (7) and achievement of GRAS status would provide the impetus to further investigate phage biocontrol.

As one chooses an antimicrobial agent, it is crucial to make sure that the selected agent remains active (8-10) during processing or in a given storage environment. Several studies reported phage biocontrol unsuccessful or with limited success. It was speculated to be due to phage inactivation (1), low multiplicity of infection (MOI) (3, 11) or intrinsically inefficient phage lysis property (12, 13). In this study, I present the data suggesting that the host growth environment could affect the outcomes of phage biocontrol. Three broad host range virulent phages (A511, Felix O1, and T7), potential candidates for biocontrol agent, were tested for their infectivity and stability under representative environmental conditions (temperature, pH, salts, presence of divalent cations). Bacterial lysis in liquid media and phage adsorption were also determined when necessary. Results showed that (i) the three phages exhibited a wide range of resistance to different environments and (ii) phage infectivity was generally correlated with the host growth; one exception of which was Felix O1 against *Salmonella* grown at low pH (4.5). Defective Felix O1 adsorption was suggested to be an attributing factor. These results would be helpful for efficient use of phage as potent biocontrol agent.

Materials and Methods

Bacterial strains, phage propagation, and purification *Listeria ivanovii* 3009, *Salmonella enterica* serovar Typhimurium DB7155, and *Escherichia coli* W3110 were used as host strains for *Listeria* phage A511 (14), *Salmonella* phage Felix O1 (15), and *E. coli* phage T7, respectively. The bacteria were aerobically grown in half-concentrated Brain Heart Infusion medium (BHI 1/2, Biolife, Milano, Italy) at 30 (*Listeria*) or 37°C (*Salmonella* and *E. coli*) in a shaking incubator (150 rpm).

Phage A511 and T7 were propagated in liquid culture as previously described (16) while Felix O1 was propagated by plate lysis and elution method, followed by PEG precipitation and CsCl gradient centrifugation as described elsewhere (17). The collected phages were dialyzed against sodium chloride - magnesium sulfate (SM) buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM $MgSO_4$) overnight and stored at 4°C. In order to measure plaque forming units (PFU), a standard soft agar overlayer

method was used (18). Final titer was adjusted to 10^{12} PFU/mL in the corresponding host strain.

Phage stability under representative environments For temperature treatment, phages (10^{10} PFU/mL) were incubated in SM buffer at various temperatures (-20, 4, 37, and 42°C) for 21 days. Phage inactivation at high temperature was also measured by incubating phages (10^8 PFU/mL) in SM buffer in a water bath (60°C). For pH studies, SM buffers with different pHs (2.5, 3.5, 4.5, 7.5, and 9.5, adjusted with concentrated HCl or 5 N NaOH), were used for the phage incubation (10^8 PFU/mL) at 4°C. For phage survival in salts environments, SM buffers with various concentrations of NaCl (0, 4, 8 and 12%, w/v) were added with phage particles (10^8 PFU/mL) and incubated at 4°C for 21 days. At designated time points (See Fig. 1, 2, and 3), phage survival was monitored by

PFU counting.

Phage infectivity determined by measuring either efficiency of plating (EOP) or bacterial lysis in liquid culture Phage infectivity was determined by measuring EOP (in this study, meaning percent PFU in the given condition compare to that in the control) in various media conditions. Exponentially growing bacteria were infected with phage (10^{2-3} PFU/plate) in molten soft agar (0.6% agar in BHI 1/2 except on the treatment with 50 mM divalent cations where LB was used due to the precipitation) which contained various pHs (4.5, 5.5, and 9.5), concentrations of NaCl (2, 4, 6, 8, and 10%, w/v), or divalent cations (Ca^{2+} or Mg^{2+}) (5, 10, and 50 mM). Plates were then incubated at 30°C for A511 or 37°C for Felix O1 and T7. Phage infectivities at different temperatures (4, 10, 30, 37, and 42°C) were also measured by using BHI 1/ 2 soft agar overlayered plates. Controls were either, 30°C for A511 and 37°C for Felix O1 and T7 for temperature, pH 7.5 for pH, without addition of NaCl for salt treatment, or no addition of divalent cations.

In bacterial lysis assay, for A511, fresh BHI 1/2 was inoculated with an overnight culture of *L. ivanovii* 3009 (1 % inoculum) and incubated at either 30 or 42°C until exponential growth phase (optical density, O.D.$_{600}$ = 0.15). In Felix O1 studies, fresh BHI 1/2 at the pH of either 4.5 or 7.5 was inoculated with an overnight culture of *Salmonella* DB7155 (1% inoculum) and incubated at 37°C to the exponential growth phase (O.D.$_{600}$ = 0.1-0.3). The corresponding phages (10^9 PFU/mL) were added and
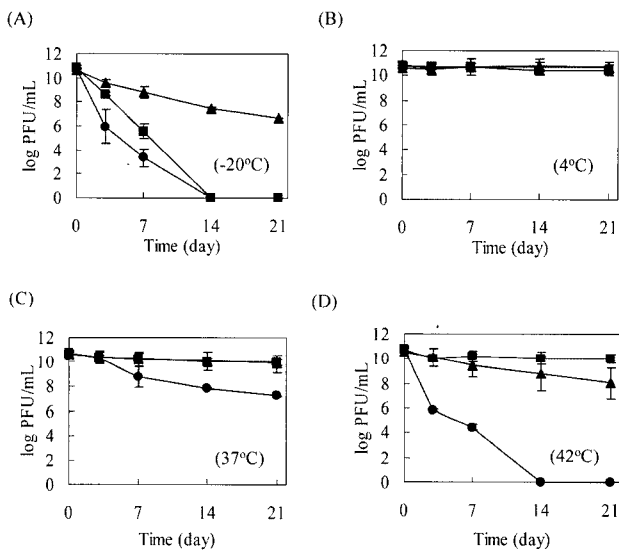

Fig. 1. Phage survival at various temperatures. Three phages (A511, Felix O1, and T7) were purified by PEG precipitation followed by ultracentrifuge in CsCl gradient and approximately 10^{11} PFU/mL of each phage was incubated at various temperatures (A, -20°C; B, 4°C; 37°C; D, 42°C). At each designated time point, phage survival was measured by soft agar layer technique. ▲, T7; ■, Felix O1; ●, A511.
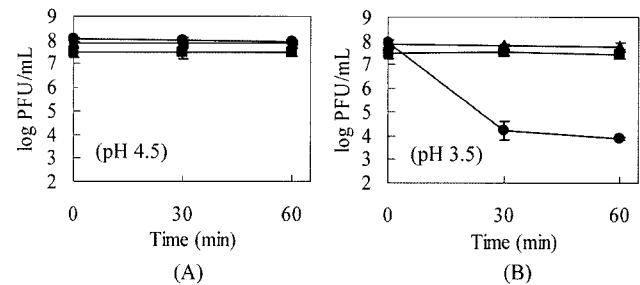

Fig. 2. Phage survival at various pHs. (A) pH 4.5 (B) pH 3.5 ▲, T7; ■, Felix O1; ●, A511.
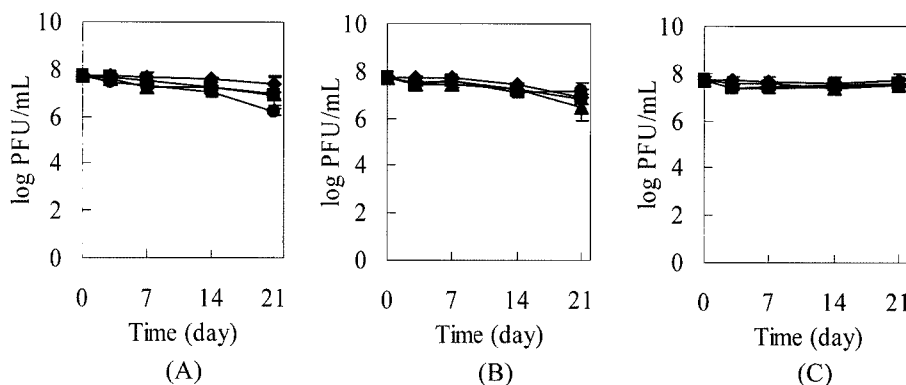

Fig. 3. Phage survival in various salt concentrations. (A) A511 (B) Felix O1 (C) T7 ◆, 0.5%; ■, 4.5%; ▲, 8.5%; ●, 12.5%.

optical densities were measured over time (See Fig. 4).

Bacteriophage adsorption study For Felix O1, exponentially growing *Salmonella* DB7155 at the pH of either 4.5 or 7.5 (6 mL, O.D.$_{600}$ = 0.2) was washed once and resuspended in SM buffer (0.25 mL, 1.5×10^8 CFU) with the respective pH of either 4.5 or 7.5. Ten μL of phage (10^6 PFU, MOI of 0.01) was added and incubated for 15 min at 37°C. For A511, *L. ivanovii* 3009 was grown at either 30 or 42°C, washed once and resuspended in SM buffer (0.25 mL, 1.0×10^8 CFU). Ten μL of phage solution (10^8 PFU, MOI of 0.01) was added and incubated for 15 min at 30, 37, or 42°C. After centrifugation at 16,100×g for 5 min, the supernatant was recovered and PFU was measured. Percent recovery was calculated by (PFU$_{supernatant}$ /PFU$_{initial}$) × 100.

Data analysis All the data are shown as average of triplicate experiments and standard deviation is indicated by error bars. The data obtained were analyzed by standard Student's *t*-test (α = 0.05).

Results and Discussion

Phage inactivation kinetics at various temperatures, pHs, and NaCl concentrations No inactivation was observed for all phages for 3 weeks at 4°C (Fig. 1B) while phage inactivation kinetics at other temperatures varied (Fig. 1A, 1C, and 1D). No inactivation was observed for all phages for three weeks at 4°C (Fig. 1B). Phage A511 was most sensitive with no detectable activity after 14 days at either -20 (Fig. 1A) or 42°C (Fig. 1D). At 37°C, A511 inactivation was slow, losing approximately 2 log PFU after 21 days (Fig. 1C). *Salmonella* phage Felix O1 showed relatively high resistance at 42°C (Fig. 1D) but was sensitive to -20°C (Fig. 1A) compared to T7. *E. coli* phage T7 was resistant at -20 and 42°C, losing 1.5 and 1.0 log PFU, respectively, after 21 days (Fig. 1A and 1D). Both T7 and Felix O1 did not lose their activities at 37°C (Fig. 1C).

At 60°C, phage A511 was again most sensitive and Felix O1 most resistant (Table 1). A511 lost approximately 4 log PFU in 5 min and 4.3 log in 10 min. The number of infective Felix O1 and T7 decreased in 10 min by only 0.5 and 1.0 log PFU, respectively, which was statistically significant ($p < 0.05$) (Table 1).

Phage survival rates were quite similar among the three tested phages when a broad range of pH was applied (pH 2.5, 4.5, 7.5, and 9.5) (only the result of pH 4.5 is shown, Fig. 2A). At pH 3.5, however, the number of infective A511 decreased about 4 log PFU within 30 min incubation, while that of either Felix O1 or T7 did not change (Fig.

Table 1. Phage inactivation at 60°C

		Incubation time (min)		
		0	5	10
Phage (log PFU/mL±SD)	A511	7.93±0.06	4.00±0.24	3.60±0.13
	Felix O1	8.33±0.04	7.99±0.18	7.74±0.10
	T7	8.22±0.02	7.58±0.11	7.32±0.03

2B). It was noticed that extended incubation (60 min) did not result in further decrease in the number of infective A511, which is consistent with previous studies (19, 20). Whether those infective phages after 30 min incubation represented a subpopulation of A511 resistant to low pH was not further investigated. All phages were not able to retain any detectable viability after 30 min incubation at pH 2.5 (data not shown). Such a dramatic inactivation of phages observed between pH 2.5 and 3.5 was also reported previously (20). All other tested pHs (4.5, 7.5, and 9.5) had no effect on phage viability within the given incubation times (only the result of pH 4.5 is shown, Fig. 2A).

High salt concentration (4, 8, and 12% NaCl) did not significantly affect viabilities of A511 (Fig. 3A), Felix O1 (Fig. 3B), or T7 (Fig. 3C) up to 3 weeks.

Overall, Listeriophage A511 was relatively sensitive to various temperatures (-20, 37, 42, and 60°C), and pH (3.5) compared to Felix O1 and T7. Broad host range *Salmonella* phage Felix O1 exhibited relatively high resistance to temperatures (37, 42, and 60°C). Throughout the experimental setup, *E. coli* phage T7 was more resistant than others except at high temperatures (42 and 60°C) in which Felix O1 revealed higher resistance than T7 (Fig. 1; Table 1). Thus different structural characteristics, T7 belonging to the family Podoviridae with short tail while A511 and Felix O1 to Myoviridae with long tail, must not be the only factor determining phage stability. The lack of clear relatedness between phage groups and their inactivation kinetics was previously observed in phages of lactic acid bacteria (19, 21, 22).

The data also undermined the evolution point of view on which it is reasonable that phage resistance could be related to the environment of host habitat. For example, *Listeira* can grow in wide range of temperatures even at 45°C and thus the least resistance of A511 in various temperatures (Fig. 1, Table 1) was intriguing. In addition, the natural habitat of *Salmonella* is speculated to be animal intestines and thus the finding that Felix O1 was most sensitive in bile salts, serum, and hydrogen peroxide (data not shown) was surprising. Taken together, one may not be able to predict phage stability under certain environmental condition based on the origin or morphological characteristics of phages. Therefore, phage inactivation kinetics needs to be evaluated individually for each phage strain.

Phage infectivity under various environments In order to investigate phage infectivity in various conditions, first, bacterial growth was monitored on soft-agar overlayered plates (Table 2). *L. ivanovii* was able to grow (form lawns) at all tested temperatures (4-42°C), in a broad range of pHs (5.5-9.5 but not of pH 4.5) or in the presence of NaCl (up to 6% but not in 8 or 10%). Growth of *Salmonella* was observed at temperatures (37 and 42°C but not at 4°C), in pH (4.5-9.5) or in the presence of NaCl (2-4% but not in 8 or 10%). Growth was not obvious for *Salmonella* at a temperature of 10°C or in the presence of 6% NaCl after 5 days incubation. *E. coli* formed lawns on agar plates at temperatures (10-42°C but not at 4°C), at pHs of 4.5-9.5 or in the presence of NaCl (2-6%, but not in 8 or 10%). Various concentrations (up to 50 mM) of divalent cations (Mg^{2+} or Ca^{2+}) did not significantly affect lawn formation

Table 2. Bacterial growth and phage infectivity (Infect.) in soft agar layered plates[1]

Temp.	4°C[2]		10°C		30°C		37°C		42°C	
	Growth	Infect.	Growth	Infect.	Growth	Infect.	Growth	Infect.	Growth	Infect.
A511	+	+++	+	+++	+	+++	+	+	+	-
Felix	-	-	-	-	NT	NT	+	+++	+	+++
T7	-	-	+	+	NT	NT	+	+++	+	+

pH	4.5[2]		5.5		7.5		9.5	
	Growth	Infect.	Growth	Infect.	Growth	Infect.	Growth	Infect.
A511	-	-	+	+++	+	+++	+	+++
Felix	+	-	+	+++	+	+++	+	+
T7	+	+	+	+++	+	+++	+	+++

NaCl	2%		4%		6%		8%[2]		10%[2]	
	Growth	Infect.	Growth	Infect.	Growth	Infect.	Growth	Infect.	Growth	Infect.
A511	+	+++	+	+++	+	+	-	-	-	-
Felix	+	+++	+	+	-	-	-	-	-	-
T7	+	+++	+	+	+	+	-	-	-	-

Cations	Mg^{2+} (mM)				Ca^{2+} (mM)			
	5 or 10		50		5 or 10		50	
	Growth	Infect.	Growth	Infect.	Growth	Infect.	Growth	Infect.
A511	+	+++	+	+	+	+++	+	+
Felix	+	+++	+	+	+	+++	+	+++
T7	+	+++	+	+	+	+++	+	+

[1]Bacterial growth was shown by positive (+) or negative (-) determination. Infectivity was determined by taking percent of PFU under the applied condition compare to that in the control. +++, 50-100% PFU compare to the control; +, 1-49%; -, <1%; NT, not tested. [2]5 days old.

(Table 2).

The EOP was measured to determine phage infectivity in various conditions. In general, phages were able to form plaques given that the host formed lawns under the given condition (Table 2). Only two exceptions were found: (i) A511 was not able to form plaques (<1% of PFU in the control) at 42°C and (ii) Felix O1 was incapable of forming plaques in low pH, 4.5. Furthermore, EOP of A511 was significantly lower (1-49% compared to the control) at 37°C, in 6% NaCl or 50 mM cations, while that of Felix O1 at 4% NaCl, pH 9.5 or 50 mM Mg^{2+}. The EOP of phage T7 was also lower at 10°C, 4 or 6% NaCl, or pH 4.5, than the control (Table 2).

In order to confirm inability of A511 at 42°C and Felix O1 in pH 4.5 to infect the host, bacterial lysis in liquid culture was tested. In liquid media, A511 was able to decrease cell densities equally efficiently either at 30 (Fig. 4A) or 42°C (Fig. 4B), indicating that A511 was, in fact, able to infect *Listeria* at both temperatures. In *Salmonella* study, Felix O1 effectively inhibited *Salmonella* growth at pH 7.5 (Fig. 4C). However, at pH 4.5, Felix O1 failed to lyse *Salmonella* (Fig. 4D) although bacteria grew at pH 4.5 with a slower growth rate (Fig. 4D) than at pH 7.5 (Fig. 4C). No difference in cell densities was observed between Felix O1-treated and non-treated *Salmonella* cultures at pH 4.5 (Fig. 4D). The comparable results were also found when organic acid, such as lactic acid, was used to adjust the pH (data not shown).

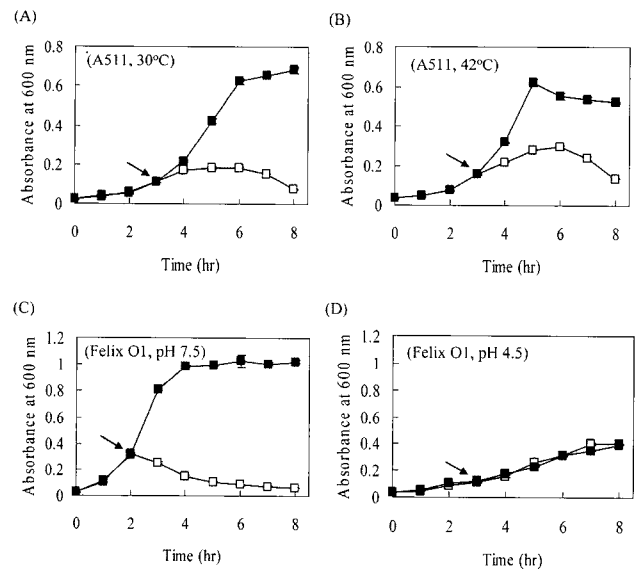As the buffer pH of 4.5 did not inactivate Felix O1 (Fig.

Fig. 4. Bacterial lysis in liquid culture. (A) A511 at 30°C, (B) A511 at 42°C, (C) Felix O1 in BHI 1/2, pH 7.5, (D) Felix O1 in BHI 1/2, pH 4.5. Arrows indicate the time point at which the corresponding phage was added. ■, no phage added; □, phage added at the level of 10^9 PFU/mL.

2), its inability to kill *Salmonella* is likely to be related to the infection process(es). In an effort to find out a possible explanation for such a failure by Felix O1 in low pH,
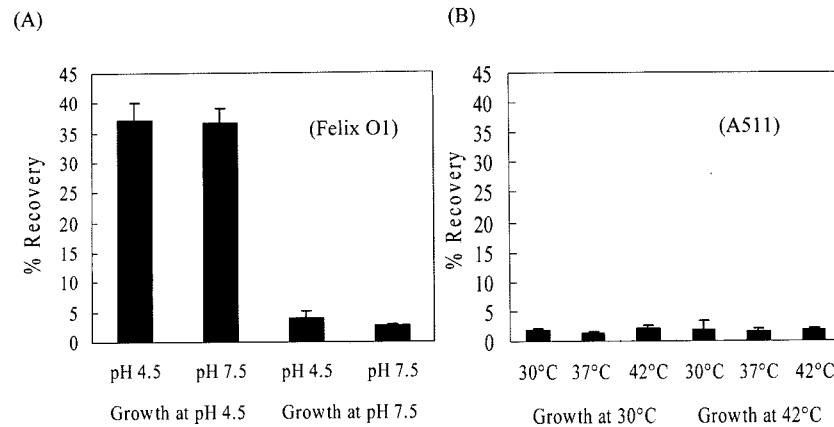
(A)   (B)

Fig. 5. Phage adsorption studies expressed in percent recovery. Salmonella was grown in BHI 1/2, pH of either 4.5 or 7.5 at 37°C, and then used for adsorption study in SM buffer, pH of either 4.5 or 7.5. Listeria was grown in BHI 1/2 either at 30 or 42°C, and then used for adsorption study in SM buffer at either 30, 37, or 42°C.

phage adsorption, the first step of phage-host interaction, was tested. When Felix O1 was incubated with Salmonella cells grown at pH 4.5 at an MOI of 0.01 (Fig. 5A), significantly higher percentage (35-40%) of Felix O1 was recovered (Fig. 5A) from the supernatant than in the control (growth at pH 7.5, 2-4%). No effect of incubation pH (4.5 or 7.5) during adsorption reaction was observed (Fig. 5A). Thus, it is clear that the exposure of Salmonella to low pH affected the bacterial host so that Felix O1 is inefficient to bind. Whether the receptor of Felix O1 such as N-acetyl glucosamine in the common core polysaccharide of Salmonella lipopolysaccharide (23) is down-regulated or modified is to be elucidated. Obviously, however, it can't be excluded that infection might be aborted in the downstream of the infection processes by host response to low pH.

No difference in A511 adsorption to the host was observed between 30 and 42°C (Fig. 5B), suggesting that the discrepancy observed between results from soft agar overlayer plate (Table 2) and liquid culture (Fig. 4B), was likely to be due to the lower EOP of A511 at 42°C and not due to defective infection.

In conclusion, based on our results and previous studies, phages seem to be quite stable particles that were resistant to a wide range of environmental conditions, but at various rates. Therefore, certain phage resistance properties need to be checked individually. More importantly, phage infectivity was, in general, well-correlated with host growth (Table 2; Fig. 4). One exception was Felix O1 at low pH, 4.5 under which phage adsorption was abolished. This observation could be very important in analyzing the outcomes of phage biocontrol. The basic information obtained in this study will be of great value to deepen the understanding of the relevant mechanisms of phage biocontrol.

Acknowledgments

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