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NOTE

Host range and influence of a cell capsule on the phage efficacy of three *Lactococcus garvieae* lytic phages

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ABSTRACT: Three lytic phages (PLgW-1, PLgY-16, and PLgY-30) were previously used for phage-typing *Lactococcus garvieae*, a bacterial pathogen of various marine fish species, and were demonstrated to be potential therapeutants for infections caused by *L. garvieae*. The morphology, host range, and efficacy of these phages have not been investigated in detail, however. The current study examined the lysis spectrum of these 3 phages against 16 different genotypes of *L. garvieae* and the influence of a bacterial capsule on phage efficacy, to aid in developing an effective treatment for lactococcosis in fish. Morphological analysis by transmission electron microscopy revealed that all 3 phages belonged to the family *Siphoviridae* and had a minor difference in morphology. These phages lysed a high proportion of their bacterial host (93.7% of the different *L. garvieae* genotypes). In addition, the efficacy of the plating assays was affected by both the phages and their bacterial host, in which phage efficacy was clearly affected by a bacterial capsule. The results of this study may be useful for developing appropriate strategies to use these phages to control various genotypes of *L. garvieae* causing disease in marine fish.

KEY WORDS: Lytic phage · Host range · Cell capsule · Efficacy · *Lactococcus garvieae* · Marine fish

INTRODUCTION

In Japan, aquaculture production has been growing. Yellowtail *Seriola quinqueradiata*, amberjack *S. dumerili*, and kingfish *S. lalandi* are the most extensively cultured fish species, and account for 58% of the total production of farmed fish (Kawanishi et al. 2006). *Lactococcus garvieae* causes one of the most serious diseases in fish of the genus *Seriola*, resulting in a significant decrease in aquaculture production (Vendrell et al. 2006). Outbreaks of this infection are caused by different bacterial genotypes which can spread to various fish species (Nishiki et al. 2011). Three lytic phages, PLgW-1, PLgY-16, and PLgY-30, have been used for phage-typing of *L. garvieae*, testing the sensitivity to several *L. garvieae* strains of unknown genotype isolated from yellowtail (Park et al. 1998), and were demonstrated to effectively protect yellowtail from infection with a single *L. garvieae* strain (Nakai et al. 1999). Thus, these phages can effectively control *L. garvieae* infection in these marine fish species if infected by different *L. garvieae* strains and can lyse their host bacteria prior to their practical application. To this end, an epidemiological

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study is required to determine the range of host bacterial species susceptible to the phages using a collection of previously isolated *L. garvieae*. This collection of *L. garvieae* included both capsulated and non-capsulated strains (Kawanishi et al. 2007), and several studies have demonstrated that the bacterial capsule was found to affect the efficacy of phage therapy (Bernheimer & Tiraby 1976, Scholl et al. 2005). Consequently, it is necessary to clarify the influence of the *L. garvieae* capsule on the ability of the phages to infect the bacteria.

The efficacy of these phages on a wide range of *L. garvieae* genotypes isolated from various marine fish species over a period between 1974 and 2012 was examined in the present study, together with the influence of the bacterial capsule on phage efficacy, in order to develop an appropriate strategy to control *L. garvieae* infections.

MATERIALS AND METHODS

Microbial strains and culture conditions

Chromosomal DNA samples of 427 *Lactococcus garvieae* strains isolated from several marine fish species between 1974 and 2012 were digested with *Sma*I. The fragments were separated using biased sinusoidal field gel electrophoresis (BSFGE), and classified into 16 bacterial genotypes based on 16 types of *Sma*I restriction patterns. The genetic similarity index between 16 genotypes obtained by dendrogram based on cluster analysis was >70% (Nishiki et al. 2011). A representative strain of each genotype was randomly selected to be used in this study (all strains in each genotype were assigned a sequential number, and a random number generator was used to select the strain from each genotype; Table 1). In addition, the strain NSS9310 (non-capsulated, KG^+ phenotype, genotype S2) was used as an indicator bacterium for these phages. *L. garvieae* isolates were preserved at –80°C, and a liquid culture medium and a solid medium (Bacto Todd Hewitt Broth [THB] and THB with agar [THA], respectively; Difco) were used to aerobically culture each bacterial strain at 25°C.

Plaque purification, lysate preparation, and bacteriophage titration

Three lytic phages were used in this study, PLgW-1 (obtained from seawater collected at a fish farm), and PLgY-16 and PLgY-30 (obtained from *L. garvieae* isolated from diseased yellowtail), previously applied for phage-typing of *L. garvieae* (Park et al. 1998). The phages were isolated and stocks of phages were prepared using the plate lysis method, described in detail previously (Hoai & Yoshida 2016). The phage titer was measured using a double-layer agar assay (Adams 1959), and average phage production was determined by performing 3 replicate experiments.

Table 1. Sources of representative *Lactococcus garvieae* strains used in this study

Genotype	Rep. strain	Year isolated	Isolated from fish species
S1	Lg2	2002	*Seriola quinqueradiata*
S2	KYS9303	1993	*S. quinqueradiata*
S3	KS9402	1994	*S. quinqueradiata*
S4	NA8706	1987	*S. quinqueradiata*
S5	KG8712	1982	*S. quinqueradiata*
S6	KG8819	1988	*S. quinqueradiata*
S7	KG32	1980	*S. quinqueradiata*
S8	SS9034	1990	*S. quinqueradiata*
S9	SS9074	1990	*S. quinqueradiata*
S10	KG9271	1992	*S. dumerili*
S11	12562	1991	*Paralichthys olivaceus*
S12	14972	1991	*P. olivaceus*
S13	AZ9834	1998	*S. quinqueradiata*
S14	12563	1991	*P. olivaceus*
S15	DKT5804	2005	*S. dumerili*
S16	EH6704	2006	*S. quinqueradiata*

Transmission electron microscopy (TEM)

To compare the morphological features of these phages, a drop of the concentrated phage suspension was placed on a glow-discharged Formvar-coated 200-mesh copper grid and allowed to adsorb for 10 min. Excess liquid was removed with a filter paper, and phage particles were negatively stained with a drop of 2% uranyl acetate for 1 min. Phage samples were examined under a JEM-2010 MX biological TEM (JEOL, Japan) operated at an accelerating voltage of 100 kV (Hoai & Yoshida 2016). Phage size was determined from an average of 10 independent measurements. Bacterial capsule presence was confirmed by TEM as described by Yoshida et al. (1997).

Slide agglutination test for confirmation of phenotype

L. garvieae isolated from fish belonging to the genus *Seriola* in Japan were divided into serologi-

cally non-agglutinating (KG^-) and agglutinating (KG^+) phenotypes using anti-KG^+ phenotype rabbit serum (Kitao 1982, Yoshida et al. 1997). KG^- strains agglutinated with anti-KG^- cell serum (but not with anti-KG^+ serum). However, KG^+ strains agglutinated with antisera to both KG^+ and KG^- (Kitao 1982). The KG^- phenotypes were correlated with the presence of a cell surface capsule (Yoshida et al. 1996). In the present study, a slide agglutination test was used to confirm the phenotype and to detect the presence of a capsule in all the representative strains of the different bacterial genotypes. Briefly, representative strains of bacterial genotypes were cultured overnight on THA at 25°C, and then a group of 3–4 colonies of each strain was thoroughly mixed with 50 µl of each antiserum using an inoculating loop. A similar experiment, in which the antiserum was replaced by a similar volume of phosphate-buffered saline (PBS), was used as a negative control (negative agglutination). The mixture was gently stirred, and visible specific clumping of bacterial cells within 5 min was considered as positive agglutination.

Host range and efficiency of plating (EOP)

To determine the host range and susceptibility of *L. garvieae* to the 3 phages, the phage lysate was diluted to 10^6 plaque-forming units (PFU) ml^{-1} and a spotting test was performed. According to Mirzaei & Nilsson (2015), the EOP assay is more suitable than the spotting test for determining host range and phage efficacy, because the spotting test often overestimates both overall virulence and host range. Therefore, the EOP assay was also used to determine both the host range and phage efficacy for all *L. garvieae* genotypes in order to develop an effective phage therapy. The phage lysate was diluted from the phage stock by 10^5 to 10^9 times. The phage titer for the *L. garvieae* strain NSS9310, the indicator bacteria, was used as the standard EOP (EOP = 1.0) against which each phage was compared. The EOP assay was repeated 3 times for each phage and 16 bacterial genotype strains were tested using the double-layer agar method. The plates were incubated overnight at 25°C, and the numbers of PFU were counted for each phage. When the 10^5 dilution did not form any plaque, a lower dilution was applied to determine the host range and EOP. For each phage, the experiment was independently performed 3 times, and the EOP was calculated (average PFU on target bacteria divided by the average PFU on host bacteria) together with the standard deviation of 3 measurements (Kutter 2009).

RESULTS

Comparison of phage morphology

The 3 phages of *Lactococcus garvieae* formed clear and small plaques of approximately 0.7–1.5 mm diameter. The morphological features of the phages obtained using TEM are shown in Fig. 1. The TEM images revealed that all the phages had a long, flexible, and non-contractile tail. The tail size of phage PLgW-1 was smaller than that of the other 2 phages (Table 2). Despite the morphological differences, all phages belonged to the family *Siphoviridae* in the

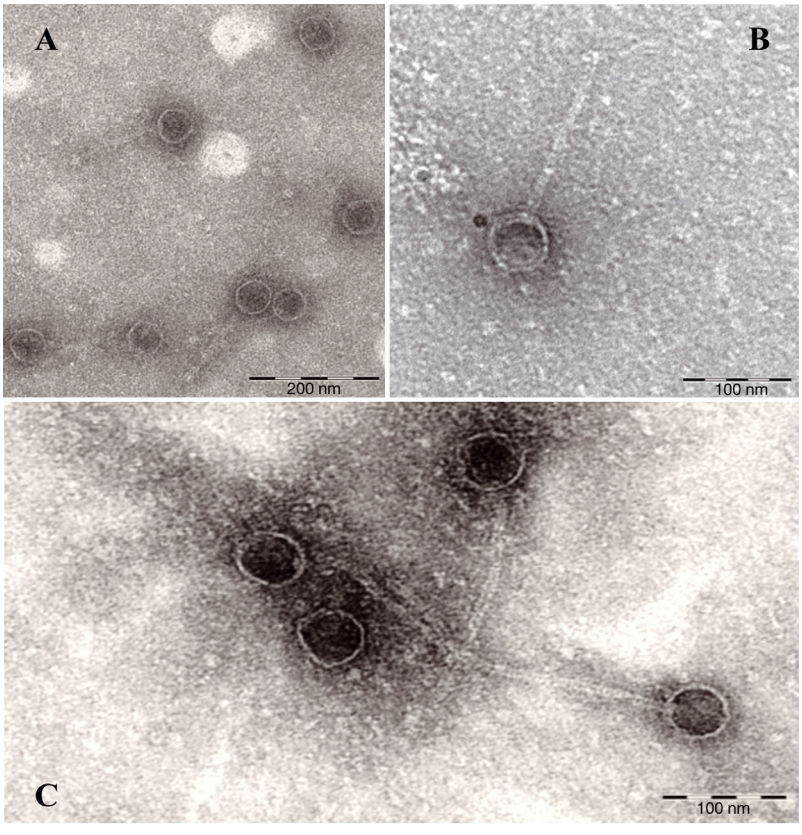

Fig. 1. Transmission electron microscopy images of 3 lytic phages negatively stained with 2% uranyl acetate: (A) PLgW-1, (B) PLgY-16, and (C) PLgY-30

order *Caudovirales*, based on the classification proposed by Ackermann (1998).

Capsule detection

Of the 16 representative bacterial genotypes of *L. garvieae*, genotypes S1, S3, S7, S9, and S12 were agglutinated with the anti-KG$^-$ serum but not with the anti-KG$^+$ serum, and so were determined as KG$^-$ genotypes, with a capsule. Other genotypes were determined as non-capsulated KG$^+$ genotypes because they were agglutinated by both the anti-KG$^+$ and the anti-KG$^-$ sera (Table 3). Non-capsulated bacteria Lg2-S and SS9074$^+$, which were mutants derived from capsulated bacterial strains Lg2 and SS9074, respectively, by sub-culturing several times on THB (Morita et al. 2011), were confirmed to be non-capsulated strains by slide agglutination (Table 3), growth characteristics (the bacteria automatically switch from dispersion to sedimentation in THB; Fig. 2A), and bacterial morphology assessed by TEM (Fig. 2B). The non-capsulated bacteria Lg2-S and SS9074$^+$ were used in the EOP assay to determine the effect of a capsule on phage efficacy.

Table 2. Morphological features of the 3 lytic phages. Measurements (nm) of phage particles derived from transmission electron microscopy. Data are mean (±SE) dimensions based on 10 phages

Phage	Head diameter	Tail length	Tail width
PLgW-1	55.6 ± 0.7	149.1 ± 1.8	9.4 ± 0.1
PLgY-16	59.7 ± 1.2	165.5 ± 1.4	10.0 ± 0.1
PLgY-30	58.4 ± 0.6	170.1 ± 2.6	10.3 ± 0.1

Table 3. Agglutination test and efficiency of plating (EOP) to various *Lactococcus garvieae* genotypes from 3 lytic phages. Values were calculated from the average of 3 replicate measurements; (–) no sensitivity. Shaded rows indicate KG$^-$ phenotype with presence of cell capsule

Bacterial genotype	Rep. strain	Agglutination test	EOP		
			PLgW-1	PLgY-16	PLgY-30
Indicator	NSS9310	KG$^+$	1.0 (2.4×10^{10})[a]	1.0 (1.2×10^{10})[b]	1.0 (2.1×10^{10})[c]
S1	Lg2	KG$^-$	5.0×10^{-3}	1.3×10^{-3}	1.1×10^{-3}
	Lg2-S	KG$^+$	1.2×10^{-1}	4.7×10^{-1}	5.2×10^{-1}
S2	KYS9303	KG$^+$	2.0×10^{-1}	6.5×10^{-1}	2.4×10^{-1}
S3	KS9402	KG$^-$	–	–	–
S4	NA8706	KG$^+$	8.4×10^{-1}	7.7×10^{-1}	6.4×10^{-1}
S5	KG8712	KG$^+$	5.2×10^{-1}	8.0×10^{-1}	4.6×10^{-1}
S6	KG8819	KG$^+$	6.4×10^{-3}	7.2×10^{-1}	2.9×10^{-1}
S7	KG32	KG$^-$	1.3×10^{-6}	3.3×10^{-5}	2.7×10^{-6}
S8	SS9034	KG$^+$	9.1×10^{-1}	9.7×10^{-1}	9.6×10^{-1}
S9	SS9074$^-$	KG$^-$	4.2×10^{-3}	4.4×10^{-3}	9.0×10^{-3}
	SS9074$^+$	KG$^+$	9.2×10^{-1}	1.1	9.2×10^{-1}
S10	KG9271	KG$^+$	1.9×10^{-3}	1.5×10^{-1}	1.1×10^{-1}
S11	12562	KG$^+$	1.5×10^{-1}	2.1×10^{-1}	3.7×10^{-1}
S12	14972	KG$^-$	1.4×10^{-2}	3.6×10^{-2}	6.4×10^{-2}
S13	AZ9034	KG$^+$	2.2×10^{-1}	3.9×10^{-1}	3.5×10^{-1}
S14	12563	KG$^+$	1.2×10^{-1}	3.1×10^{-1}	3.4×10^{-1}
S15	DKT50804	KG$^+$	4.8×10^{-3}	3.1×10^{-1}	1.8×10^{-1}
S16	EH6704	KG$^+$	8.2×10^{-1}	5.0×10^{-1}	8.0×10^{-1}

[a,b,c]Phage titer (PFU ml^{-1}) for indicator bacteria (*L. garviae* strain NSS9310) which was used as the baseline EOP (EOP = 1) against phages PLgW-1, PLgY-16 and PLgY-30, respectively

Host range, EOP, and effect of capsule on phage efficacy

Antagonistic activity of the phage on the bacteria, indicated by a transparent region on the bacterial lawn in the region where the phage suspension was spotted, was used to determine the host range of the 3 phages in the spotting test. Phages PLgY-16 and PLgY-30 were found to be virulent to 15 out of 16 representative bacterial genotypes of *L. garvieae* tested (genotype S3 was not sensitive to either phage). The antagonistic activity of PLgW-1 on the bacterial lawns of the genotypes S1, S6, S10, and S15 was low, and no antagonistic activity was observed on the bacterial lawn of genotype S3. In the EOP assay using 16 genotypes of either capsulated or non-capsulated bacteria (Table 3), a correlation was observed between phage efficacy and the presence of a bacterial capsule, and the EOP was higher with non-capsulated than with capsulated bacteria. In addition, the sizes of plaques formed on the bacterial lawns of non-capsulated bacteria were larger than those that formed with capsulated bacteria (Fig. 3). Thus, phage production was higher in non-capsulated bacteria compared with capsulated bacteria. In general, phage production is affected by the bacterial genotype due to external features, such as the number of receptors and the cell wall (Rakhuba et al. 2010). In the present study, phage production was affected by the capsule because bacterial strains Lg2 and SS9074 were capsulated, whereas Lg2-S and SS9074$^+$ were not. Thus, this study again confirmed that phage infectious efficacy is affected by a capsule.

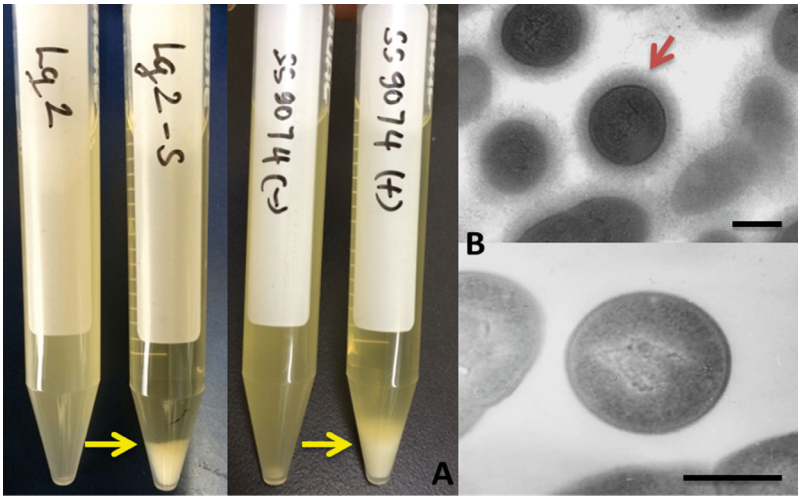

Fig. 2. (A) Sedimentation of non-capsulated *Lactococcus garvieae* strains (Lg2-S and SS9074^{+}) in Bacto Todd Hewitt Broth (THB) derived from capsulated bacteria (Lg2 and SS9074^{-}) after sub-culturing several times on THB with agar (THA) (yellow arrows), and (B) confirmation of capsulated (upper photo; red arrow pointing to the capsule) and non-capsulated (lower photo) bacterial strains by transmission electron microscopy. Scale bars = 1 µm

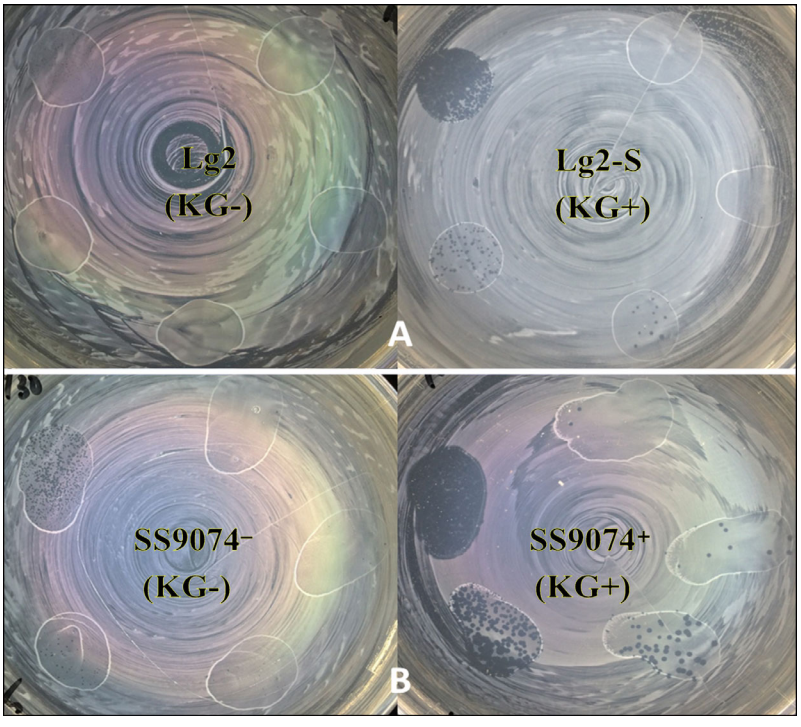

Fig. 3. Effects of capsulated and non-capsulated *Lactococcus garvieae* on ability of phage to infect and replicate. Phage infection and replication observed on lawns of (A) capsulated strain Lg2 (left) and non-capsulated strain Lg2-S (right) in the spotting test with phage PLgW-1; and (B) capsulated strain SS9074^{-} (left) and non-capsulated strain SS9074^{+} (right) in the spotting test with phage PLgY-30. Phage suspensions were applied in a series of dilutions from 10^{6} to 10^{2} PFU ml^{-1} (from the top left corner of each panel, in a counterclockwise direction). KG^{-}: non-agglutinating phenotype; KG^{+}: agglutinating phenotype

DISCUSSION

The bacterial capsule has been shown to affect phage adsorption and prevent phage infections in various bacterial species such as *Escherichia coli* (Scholl et al. 2005), *Staphylococcus* sp. (Ohshima et al. 1988), and *Streptococcus pneumoniae* (Bernheimer & Tiraby 1976). This phenomenon was also observed in *Lactococcus garvieae*, a pathogen infecting various marine fish. Capsulated bacteria of various genotypes affected the efficacy of all 3 phages in the present study, with the EOP of non-capsulated bacteria higher than that of capsulated bacteria by approximately 10^{2}–10^{6} times (Table 3). The EOP assay is known to establish the efficacy of phages (Mirzaei & Nilsson 2015). However, even if the same phage was used, the plaques formed on the bacterial lawn differed according to the existence of a capsule. In other words, the sizes of the plaques formed on lawns of non-capsulated bacteria were larger (approximately 1–2 mm in diameter) and clearer than those formed on the lawns of capsulated bacteria (<0.3 mm in diameter). This is probably because the capsule reduced phage adsorption. This phenomenon was also confirmed by comparing the morphology and size of the plaques formed on the same bacterial lawn before and after removing the capsule by sub-culturing several times (Fig. 3). Thus, comparison of phage efficacy and phage production between non-capsulated and capsulated bacteria are probably not reflected accurately in the evaluation of the EOP assay results.

Bacteriophage resistance mechanisms have been investigated in detail, including the prevention of phage adsorption, blocking of phage receptors, production of extracellular matrix, prevention of phage DNA entry, excision of phage nucleic acids, and the phage abortive infection system (Labrie et al. 2010). In the present study, although the 3 phages investigated

have the same host range, the results of the spotting test and EOP assay revealed that PLgW-1 was less infectious to some bacterial genotypes, including both capsulated and non-capsulated genotypes, compared to PLgY-16 and PLgY-30. Therefore, regarding the process of infection, PLgW-1 was probably more affected by the capsule as well as other antiviral mechanisms of *L. garvieae* genotypes than PLgY-16 and PLgY-30. Moreover, the capsulated S3 bacterial genotype was not infected by any of the phages, and the EOP of the phages was significantly lower for the capsulated S7 bacterial genotype than for other genotypes. This finding implies that the resistance mechanisms against phage infection were probably more complicated for the S3 and S7 bacterial genotypes than for the other genotypes.

Although a bacterial polysaccharide capsule was demonstrated to be effective against phage adsorption, all 3 lytic phages examined here passed through the capsule barrier of *L. garvieae*, penetrating the bacterial cells, lysed the host bacteria, and formed plaques. It may be true that the capsule provides a selective advantage for phages to improve their ability to degrade the barrier. For example, many phages specific to *E. coli* were reported to possess the capacity to counter the polysaccharide barrier using their tail proteins that possess depolymerization activity (Nimmich 1997). Many phages were also reported to use bacterial pili, flagella, and slime polysaccharides as receptors (Rakhuba et al. 2010). It is therefore important to study the interactions between the 3 lytic phages in the present paper and *L. garvieae* strains in more detail to understand the mechanisms involved in their activity. Further studies are also needed relating to the resistance mechanisms of *L. garvieae*, and determination of the receptors for these 3 phages are needed to enhance their antimicrobial potential.

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