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A LYTIC PODOPHAGE SPECIFIC TO FISH PATHOGENIC *EDWARDSIELLA TARDA*

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ABSTRACT

A lytic bacteriophage specific to *Edwardsiella tarda*, a fish pathogen, was isolated from fish culturing pond water. The bacteriophage, designated P8, was highly specific to the species *E. tarda*. It was tolerant to high temperature up to 70°C and to pH from 4 to 11. As studied by transmission electron microscope, the bacteriophage was a short-tail bacteriophage. The genome of bacteriophage P8 was found to be double stranded DNA, indicated by its sensitivity to *Eco*RI. Based on its morphology and genome, bacteriophage P8 was classified as a podophage. Five major proteins of bacteriophage P8 (70, 45, 35, 37 and 23 kDa) were detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis. This study shows that bacteriophage P8 had specificity, host range and stability favoring its use as a therapeutic agent against *E. tarda* infection in aquaculture.

Keywords: bacteriophage, *Edwardsiella tarda*, fish pathogen, *Podoviridae*

INTRODUCTION

Edwardsiella tarda is a Gram-negative rod shaped bacterial species causing a fish disease, edwardsiellosis, in various economically important fish species (Minagawa *et al.* 1983; Nougayrede *et al.* 1994; Castro *et al.*, 2008; Rattanachaikunsopon and Phumkhachorn 2010). Consequently, its infection can lead to serious economic losses in aquaculture worldwide. Major symptoms of edwardsiellosis include septicemia and systemic inflammation. Death can occur in severe cases. Severity of the disease can be influenced by several factors especially inappropriate culturing conditions.

Antibiotics are commonly used to cure *E. tarda* infection in fish farming. Although they are effective, they have many negative impacts. The improper use of antibiotics can increase the tendency of the emergence of antibiotic resistant strain and the accumulation of antibiotics in fish and environments. Therefore, many reports have recently proposed safe and effective alternatives to treat *E. tarda* infection in fish. Among them, bacteriophage therapy is one of the promising alternatives.

Bacteriophages (or phages) are bacterial viruses that can be found almost everywhere in the environments (Othman *et al.*, 2008; Sadik *et al.*, 2016). They generally have high specificity to their hosts. Many of them are active against only a specific strain. This property makes bacteriophages become candidates for use as biocontrol agents for treating *E. tarda* infection in fish because they affect only their target bacteria without disturbing beneficial microorganisms in fish. Several bacteriophages have been reported to specifically inhibit fish pathogens such as *Aeromonas hydrophila* (Merino *et al.*, 1990), *Aeromonas salmonicida* (Kim *et al.*, 2015), *Flavobacterium columnare* (Laanto *et al.*, 2011), *Flavobacterium psychrophilum* (Stenholm *et al.*, 2008), *Lactococcus garvieae* (Ghasemi *et al.*, 2014), *Pseudomonas aeruginosa* (Khairnar *et al.*, 2013), *Pseudomonas plecoglossicida* (Park *et al.*, 2000) and *Streptococcus agalactiae* (Phumkhachorn and Rattanachaikunsopon, 2014).

This study aimed to isolate a bacteriophage specific to *E. tarda* from fish culturing pond water and to partially characterize the bacteriophage in some aspect including host range, thermal and pH stability and protein composition. Furthermore, the bacteriophage was also classified based on its structure and genetic material.

MATERIALS AND METHODS

Bacteria: *E. tarda* PP00124 was used as the principle bacterial host for bacteriophage detection and isolation (Rattanachaikunsopon and Phumkhachorn, 2010). The bacteria presented in Table 1 were used for the determination of bacteriophage host range. Brain Heart Infusion (BHI) medium were used to culture all of the bacteria at 30ºC. The stock cultures of bacteria were prepared in BHI broth supplemented with glycerol (20% v/v) and stored at -80ºC

Bacteriophage detection: Water samples used for the detection of a bacteriophage specific to *E. tarda* PP00124 were collected from twelve different fish culturing ponds in Ubon Ratchathani province, Thailand. Ten mL of each water sample was centrifuged for 10 min at 3,500 x g to precipitate coarse contaminants and then filtered through a membrane filter (0.45 μm pore size). The mixture of 4.9 mL of the filtrate and 0.1 mL of the log phase *E. tarda* PP00124 culture (approximately 10^8 colony forming unit (CFU/mL) were

added to 5 mL of double strength BHI (2X BHI) broth, incubated for 24 h at 30°C and then centrifuged for 10 min at 3,500 xg. The collected supernatant was filtered through a membrane filter (0.45 μm pore size) to obtain the filtrate, called tested filtrate. It was examined for the presence of bacteriophage activity against *E. tarda* PP00124 by spot test method as previously described (Phumkhachorn and Rattanachaikunsopon, 2014). The tested filtrate that produced the clear lysis zone, indicating the presence of lytic bacteriophage, was subjected to bacteriophage isolation.

Bacteriophage isolation: Bacteriophage isolation was performed by using plaque assay as described by Phumkhachorn and Rattanachaikunsopon (2015). The resulting product from this step was bacteriophage suspension.

Examination of bacteriophage host range: The lytic activity of a bacteriophage against the bacterial strains listed in Table 1 was examined by the spot test. The experiment was performed three times for each bacterial strain.

Thermal and pH stability test: To examine the thermal stability of a bacteriophage, 900 μL of BHI broth was preheated to a desirable temperature, ranging from 50 to 90 °C. Then, 100 μL of bacteriophage suspension (a final concentration of 10^6 PFU/mL) was inoculated into each of the preheated BHI broth and incubated for another 30 min at the assigned temperatures. Immediately after heating, the samples were put on ice for 10 min. The residual titers of all samples were determined by the plaque assay. This experiment was repeated three times.

To examine the pH stability of a bacteriophage, 1 mL of bacteriophage suspension (10^6 PFU/mL) was incubated in phosphate buffer (pH 2 to 12) for 2 h at 30°C. Immediately after incubition, the pH of phosphate buffer was adjusted to 7. The residual titers of all samples were determined by the plaque assay. This experiment was repeated three times.

Bacteriophage morphology study: A 5 μL aliquote of the bacteriophage suspension was dropped on a copper grid and stained with 5 μL of 2% uranyl acetate. The stained bacteriophage was examined by Philips EM300 electron microscope at 80 kV accelerating voltage. The size of bacteriophage was estimated from five measurements.

Analysis of bacteriophage genome: Bacteriophage genome was extracted by using PureLink Viral RNA/DNA Mini Kit (Invitrogen, USA) and digested by *Eco*RI, nuclease S1 and RNase A (Sigma-Aldrich, USA). The digested genome was analyzed by agarose gel electrophoresis using 0.8% agarose gel.

Analysis of bacteriophage proteins: Bacteriophage proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) as described previously (Phumkhachorn and Rattanachaikunsopon, 2010).

RESULTS AND DISCUSSION

Bacteriophage detection and isolation: In this study, we intended to find an *E. tarda* bacteriophage from fish culturing pond water due to the fact that bacteriophages and their specific host bacteria generally inhabit in the same environments. Previously, several bacteriophages specific to fish pathogens were isolated from fish culturing pond water such as those specific to *E. tarda* (Hsu *et al.*, 2000), *P. plecoglossicida* (Park *et al.*, 2000) and *S. agalactiae* (Phumkhachorn and Rattanachaikunsopon, 2014).

Of the twelve tested filtrates prepared from different fish culturing pond water samples, only one tested filtrate was shown by the spot test method to produce a clear lysis zone on the lawn of *E. tarda* PP00124, indicating the presence of a lytic bacteriophage in the tested filtrate. The bacteriophage was isolated from the tested filtrate by three rounds of plaque assay with a single plaque. The isolated bacteriophage was designated bacteriophage P8.

Bacteriophage host range: Lytic activity of bacteriophage P8 against different bacterial strains was studied by spot test. Of all 13 bacterial strains tested, only the bacteria in the species *E. tarda* (*E. tarda* ATCC 15947 and *E. tarda* ATCC 23685) were sensitive to the bacteriophage (Table 1). In contrast, the other bacteria were tolerant to the bacteriophage. Since the lytic activity of bacteriophage P8 was limited only to the species *E. tarda*, it could be considered as a bacteriophage with a narrow host range.

The narrow host range of bacteriophage P8 makes it a potential therapeutic agent against *E. tarda*. It is harmless not only to the eukaryotic hosts undergoing bacteriophage therapy but also to beneficial normal flora of the hosts. Although this characteristic of bacteriophage P8 may cause limitation in its use, this problem can be mitigated by using the bacteriophage in combination with other suitable bacteriophages to broaden the inhibitory spectrum. Bacteriophage cocktails have been reported to be effective against a number of fish pathogens including *Aeromonas salmonicida* (Rodgers *et al.*, 1981) and *Flavobacterium psychrophilum* (Stenholm *et al.*, 2008).

Table 1: Host range of bacteriophage P8

Bacteria	Lysis by phage
Aeromonas hydrophila DMST 4997	-
Aeromonas sobria DMST 1615	-
Escherichia. coli ATCC 25922	-
Edwardsictaluri ATCC 33202	-
Edwardsiella tarda ATCC 15947	+
Edwardsiella tarda ATCC 23685	+
Edwardsiella hoshinae ATCC 33379	-
Flavobacterium columnare ATCC 49512	-
Klebsiella pneumoniae ATCC 27736	-
Plesiomonas shigelloides DMST 3394	-
Pseudomonas fluorescens DMST 0793	-
Shigella dysenteriae ATCC 29026	-
Streptococcus agalactiae DMST 4314	-

Thermal and pH stability of bacteriophage: The thermal stability of bacteriophage P8 was studied by placing the bacteriophage at different temperatures 50°C to 90°C for 30 min. No significant change in bacteriophage titer was observed when the bacteriophage was treated at 50°C. However, the bacteriophage titer was found to drop from about 6 log PFU/mL to about 3 and 2 log PFU/mL after heating the bacteriophage at 60 and 70°C, respectively. Completely elimination of the bacteriophage was observed at 80 and 90°C (Table 2).

Table 2: Thermal stability of bacteriophage P8

Temperature (°C)	Initial phage titer (log PFU/mL)	Final phage titer (log PFU/mL)
50	6.03	6.05
60	6.07	3.11
70	6.02	1.92
80	6.06	ud
90	6.08	ud

ud = undetectable

The pH stability of bacteriophage P8 was studied by incubating the bacteriophage for 2 hr. at pH 2 to pH 12. At pH 4 to pH 11, the bacteriophage was still active. On the other hand, it was completely inactive at pH 2, 3 and 12 (Table 3).

Table 3: pH stability of bacteriophage P8

pH	Initial phage titer (log PFU/mL)	Final phage titer (log PFU/mL)
2	6.01	ud
3	6.10	ud
4	6.08	4.03
5	6.12	4.95
6	6.14	5.69
7	6.04	5.92
8	6.03	5.42
9	6.02	5.51
10	6.03	4.64
11	5.96	4.23
12	6.01	ud

ud = undetectable

The results demonstrated that bacteriophage P8 was stable at high temperature (up to 70°C) and over a broad pH range (4-11). These characteristics may allow the bacteriophage to be used in broad application and in the environments having fluctuated temperature and pH.

Bacteriophage morphology, genome and proteins: Bacteriophage P8 was a tailed bacteriophage as studied by transmission electron microscope (TEM). It had an isometric head of 87 ± 6.2 nm in diameter and a short tail of about 12 ± 1.4 nm in width and 20 ± 1.7 nm in length. No collar, baseplate and tail fibers were observed (Figure 1).

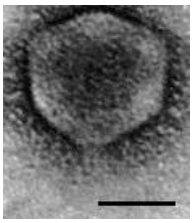

Figure 1: Transmission electron micrograph of bacteriophage P8. Bar = 50 nm

The genome of bacteriophage P8 was extracted and subjected to digestion analysis with *Eco*RI, nuclease S1 and RNase A. The digestion pattern shown in Figure 2 clearly demonstrated that the genome of bacteriophage P8 was digested by *Eco*RI but not by nuclease S1 and RNase A.

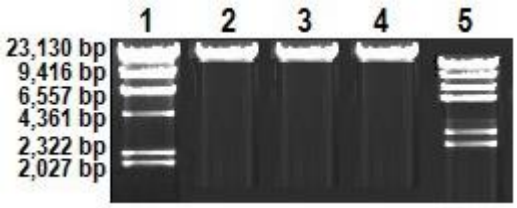

Figure 2: The digestion analysis of bacteriophage P8 genome. 1 = lambda DNA digested with *Hin*dIII marker; 2 = uncut genome; 3, 4 and 5 = genome cut with nuclease S1, RNase A and *Eco*RI, respectively

As studied by SDS PAGE, five major protein bands (70, 45, 35, 37 and 23 kDa) were detected (Figure 3). These proteins are likely to be major structural proteins of bacteriophage P8.

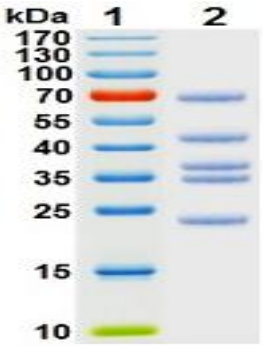

Figure 3: Protein composition of bacteriophage P8. 1 = Page Ruler Prestained Protein Ladder and 2 = bacteriophage proteins

Analysis of bacteriophage P8 morphology and genome provides necessary information for its classification. The genome of bacteriophage P8 was concluded to be double stranded DNA due to its degradability by the restriction enzyme *Eco*RI and its tolerance to nuclease S1 and RNase A. The morphology of bacteriophage P8 as studied by TEM suggested that it was a tailed bacteriophage with an isometric head and a short tail. Based on these two characteristics, bacteriophage P8 can be classified in the family *Podoviridae* (Ackermann, 2003). Tailed bacteriophages are placed in the order *Caudovirales* that can be divided by tail structures into 3 families including *Siphoviridae* (having a long, noncontractile tail), *Myoviridae* (having a long, contractile tail) and *Podoviridae* (having a short tail). Apart from bacteriophage P8, several bacteriophages specific to fish bacterial pathogens are found to be podophages such as bacteriophages PPpW-4 (Park *et al.*, 2000), FpV-2, FpV-4v (Stenholm *et al.*, 2008), WP-1, WWP-2 and SP-2 (Ghasemi *et al.*, 2014). However, such bacteriophages are not restricted to the family *Podoviridae*, many of them are members in the families *Myoviridae* and *Siphoviridae*. Bacteriophages PPpW-3 (Park *et al.*, 2000), PAS-1 (Kim *et al.*, 2012), FpV-14 and FpV-19 (Stenholm *et al.* 2008) are examples of myophages while bacteriophages S14 (Phumkhachorn and Rattanachaikunsopon, 2014), VpKK5 (Lal *et al.*, 2016), FpV-7, FpV-9 and FpV-10 (Stenholm *et al.*, 2008) are examples of siphophages.

CONCLUSION

In this study, a new bacteriophage P8 specific to fish pathogenic bacteria *E. tarda* was isolated and partially characterized. The results from this study provide preliminary data useful for designing a rational bacteriophage control strategy for *E. tarda* infection. However, further study on bacteriophage P8 is required in order to develop the proper approach to control *E. tarda* infection. In addition, the isolation and characterization of more *E. tarda* bacteriophages is now going on in our laboratory. These bacteriophages will be used together with bacteriophage P8 as bacteriophage cocktails to broaden inhibitory spectrum against many strains of *E. tarda*.

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