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A Lytic Bacteriophage with Potential for Inactivation of a Fish Pathogenic *Streptococcus agalactiae*

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Bacteriophage S14 specific to *Streptococcus agalactiae* PS01, a fish pathogenic strain, was isolated from fish pond water. It was a lytic bacteriophage that could lyse all four strains of *S. agalactiae* used in this study. It was tolerant to high temperature (up to 70°C), to a wide range of pH (3-11), and to antimicrobial chemicals commonly used in aquaculture. One-step growth curve showed that the bacteriophage had latent and burst periods of 40 and 160, respectively and burst size of 145 PFU/infected cell. Transmission electron microscopy reveals that the bacteriophage had an icosahedral head of 52 ± 4.6 nm in diameter and a noncontractile tail of 12 ± 0.4 nm in width and 210 ± 11.8 nm in length. Its nucleic acid was double stranded DNA because it was digested by *Eco*RI, but not by RnaA and nuclease S_1. Based on its morphology and genome, the bacteriophage S14 was likely to be a member of the family *Siphoviridae*. This study showed that bacteriophage S14 had specificity, host range and stability favoring its use as a therapeutic agent against *S. agalactiae* infection in aquaculture.

Keywords: Bacteriophage, fish pathogen, *Streptococcus agalactiae*.

Bacterial infection is a major problem causing heavy loss to fish farmers worldwide. Among bacterial fish pathogens, *Streptococcus agalactiae*, the Group B *Streptococcus*, is an important pathogen causing significant mortalities among numerous wild and cultured fish species, including menhaden (*Brevoortia patronus*)[1], bullminnows (*Fundus grandis*)[2], striped bass (*Morone saxatilis*)[3], mullet (*Liza klunzingeri*)[4], seabream (*Sparus auratus*)[4] and tilapia (*Oreochromis niloticus*)[4-6]. The clinical signs of *S. agalactiae* infected fish include loss of appetite, swimmimg abnormalities, unilateral or bilateral exophthalmia, corneal opacity and external hemorrhages. The internal organs of the affected fish show many changes such as spleen enlargement, ascites as well as pale liver discoloration[7]. Recently, *S. agalactiae* has become an important pathogen affected tilapia farms in Asia and the Americas. It is also the pathogen responsible for much of the mortality of farmed tilapia in Thailand in recent years[8].

The approach commonly used to control *S. agalactiae* infection in tilapia commercial farms is the use of antibiotics, especially oxytetracycline and erythromycin[9]. However, concern on adverse effects of antibiotics has been increasing due to the fact that they can lead to the development of drug resistant bacteria thereby reducing drug efficacy[10]. Moreover, the accumulation of antibiotics in the environment and in the fish can cause potential risk to consumers and to the environment[11,12]. Therefore, the need for an alternative approach to control *S. agalactiae* infection in tilapia farming is receiving increased attention. One of potential alternatives is the therapeutic use of bacteriophage or bacteriophage therapy.

Bacteriophages are viruses that infect bacteria. Their prominent characteristics are the specificity to their host bacteria. Lytic

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bacteriophage is the type of bacteriophage normally use in bacteriophage therapy because it can infect its specific bacterial host, produce its progeny inside the host cell, and kill the host to release the newly produced bacteriophages that can in turn generate infect the host. Successful uses of bacteriophage therapy to control bacterial infectious diseases have been reported since 1980s in both laboratory and farmed animals[13-15]. Attempts to apply bacteriophages in the control of aquatic animal bacterial pathogens have been increasing recently. A number of bacteriophages have been isolated for potential use in bacteriophage therapy against important aquatic animal pathogens such as *Aeromonas salmonicida*[16,17], *Flavobacterium psychrophlum*[18], *Lactococcus garvieae*[19], *Pseudomonas plecoglossicida*[20,21], *Vibrio harveyi*[22-25], *Vibrio parahaemolyticus*[17].

This study aimed to isolate and characterize a lytic bacteriophage that infects a *S. agalactiae* strain virulent to tilapia and to investigate its lytic property toward its host bacterium under controlled conditions in the laboratory. The study thus provides useful information for further use of the bacteriophage in controlling *S. agalactiae* infection in tilapia.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Bacterial strains used in this study are listed in Table 1. *S. agalactiae* PS01, isolated from diseased tilapia cultured in a fish farm in Ubon Ratchathani Province, Thailand, was used as a host strain for bacteriophage isolation and detection. The rest of the bacteria were used in bacteriophage host range determination. All of the bacterial strains except bacteria in the genus *Vibrio* were grown in Brain Heart Infusion (BHI) medium at 30°C. *Vibrio* species were grown in BHI medium containing NaCl at the final concentration of 1.5% (w/v) at 30°C. Bacterial stock cultures were stored as frozen cultures at -80°C in appropriate culturing media supplemented with 15% (v/v) glycerol. Throughout the experiments, strains were subcultured every 2 weeks on appropriate agar media and kept at 4°C. Before use, liquid cultures prepared from a single colony were transferred twice into appropriate liquid media and incubated at 30°C.

Bacteriophage isolation and detection and host range study

Twenty water samples used for bacteriophage isolation were collected from different tilapia culturing ponds in Ubon Ratchathani Province, Thailand. Ten ml of each sample was centrifuged at 3,500 xg for 10 min and the supernatant was passed through a 0.45-¼m-pore-size membrane filter (SartoriusAG, Goettingen, Germany). Five ml of the filtrate was added to equal volume of double strength BHI broth containing 10^6 CFU of *S. agalactiae* PS01 cells. After incubation at 30°C for 24 h, the culture was centrifuged at 3,500 xg for 10 min and the supernatant was passed through a 0.45-¼m-pore-size membrane filter. The filtrate was examined for the presence of lytic bacteriophage by using spot test method described by Lu *et al*.[26] with some modifications. Five ml of BHI soft agar (0.5% agar, prewarmed to 60°C) was seeded with 0.1 ml of a log phase culture of *S. agalactiae* PS01, mixed thoroughly, and poured onto a BHI agar plate. The plate was left to stand at room temperature for 30 min to allow the top agar to solidify. After solidification of the BHI soft agar, 10 ¼l of the filtrate was spotted onto the top agar layer. The plate was left at room temperature for 30 min to allow the filtrate to be well absorbed through the agar, and then incubated at 30°C for 24 h. A clear zone on the plate, resulting from the lysis of host cells, indicated the presence of lytic bacteriophage.

The spot test method was also used to examine the bacteriopahge host range by using bacterial strains listed in Table 1 as indicator organisms.

Bacteriophage purification

Bacteriophage purification was carried out following the agar overlay method described by Yoon *et al*.[27] with some modification. After appropriate dilution of phage containing sample with phosphate buffered saline (pH 7.0), 0.1 ml aliquots were added to 0.1 ml of log phase culture containing *S. agalactiae* PS01 cells at the concentration of 10^6 CFU/ml. The mixture was incubated for 15 min at 30°C, mixed with 5 ml of soft BHI agar (0.5% agar, prewarmed to 60°C) and poured onto the surface of a BHI agar plate. The plate was incubated at 30°C for 24 h to allow plaques to be formed. A single plaque was picked from the lawn of *S. agalactiae* PS01, inoculated

into phosphate buffered saline (pH 7.0) followed by dilution and re-plating for two rounds of further single plaque isolation. The final single plaque isolate was picked and transferred into a tube containing 5 ml of log phase culture containing *S. agalactiae* PS01 cells at the concentration of 10^6 CFU/ml. The tube was then incubated at 30°C for 4 h, centrifuged at 3,500 xg for 10 min and filtered using a 0.45-¼m-pore-size membrane filter. The resulting filtrate containing purified bacteriophage was called bacteriophage lysate.

The agar overlay method was also used to determine bacteriophage titer in plaque forming units per ml (PFU/ml). The number of plaques used to calculate bacteriophage titer was obtained from the plates containing a lawn of *S. agalactiae* PS01 with approximately 30-300 plaques.

Bacteriophage stability

Bacteriophage stability to various temperatures (50, 60, 70, 80 and 90°C) was examined. A microcentrifuge tube containing sterile deionized water was preheated to a desirable temperature. The bacteriophage lysate was added into the preheated water to obtain a final concentration of 10^6 PFU/ml. After heating at the assigned temperatures for 30 min, the solutions were placed in an ice-warm bath. The residual bacteriophage was counts using the agar overlay method as mentioned above and reported as percentages of the initial bacteriophage counts.

Bacteriophage stability was also examined at various pH values. The bacteriophage lysate at the final concentration of 10^6 PFU/ml was incubated at 30°C for 2 h in phosphate buffered saline adjusted in steps of 1 pH unit from pH 2 to 12 using HCl or NaOH as required. After incubation, bacteriophage titer was determined by the agar overlay method and reported as percentages of the initial bacteriophage counts.

Bacteriophage stability to antimicrobial chemicals commonly used in aquaculture was examined as follows. The chamicalas (all from Sigma-Aldrich, St. Louis, MO, USA) and their final concentrations used in this study were malachite green (0.1 ¼g/ml), formalin (30 ppm), sodium hypochlorite (100 ppm), 50% Benzalkonium chloride (1 ppm), 10% iodine (2 ppm), hydrogen peroxide (0.0001%) and oxytetracycline (10 ppm). Each of the chemicals was added to the bacteriophage lysate (10^6 PFU/ml) to obtain the predetermined concentration. After 24 h of incubation at 30°C, the surviving bacteriophage was counted by the agar overlay method and reported as percentages of the initial bacteriophage counts.

One-step growth study

Ten ml of log phase culture of *S. agalactiae* PS01 was harvested by centrifugation at 10,000 xg for 10 min and suspended in 5 ml of fresh BHI broth to obtain a final concentration of 10^8 CFU/ml. To this suspension, 5 ml of the bacteriophage lysate was added in order to have a MOI of 0.1 and the bacteriophage was allowed to adsorb for 30 min at 30°C. The mixture was then centrifuged at 10,000 xg for 10 min and the pellet was suspended in 10 ml of fresh BHI broth. Samples were taken every 20 min over a period of 3 h and immediately tittered by the method described earlier. Latent period, burst time, and burst size were calculated from the one-step growth curve.

Bacteriophage morphology examination

The transmission electron microscopy (TEM) was used to examine bacteriophage morphology. The bacteriophage to be studied by TEM was prepared using the method of Watanabe *et al.*[28] with some modifications. A 500 ml of log phase culture of *S. agalactiae* PS01 was infected with 5 ml of the bacteriophage lysate to obtain a MOI of 0.1. After incubation at 30°C for 24 h, the infected bacterial culture was centrifuged at 3,500 xg for 10 min. The supernatant was collected and subjected to centrifugation at 4°C with a 70.1Ti rotor at 28500 × *g* for 1 h in a Beckman L-80 ultracentrifuge (Beckman, Palo Alto, CA, USA). The resulting pellets were suspended in 5 ml of bacteriophage buffer (20 mM Tris-HCl pH 7.4, 100 mM NaCl, 10 mM MgSO4). A purified bacteriophage was recovered after centrifugation at 3,500 x*g* for 10 min and the supernatant was passed through a 0.45-¼m-pore-size membrane filter. The purified bacteriophage was stored at 4°C until use.

A 5-¼l aliquote of purified bacteriophage suspension was spotted on top of a Formvar-coated copper grid (Proscitech, Brisbane, Queensland, Australia) and allowed to adsorb for 5 min at room temperature. The bacteriophage was stained by adding 5 ¼l of 2% sodium phosphotingstate (pH 7.2). After 4 min, excess stain was removed and the grid was allowed to air dry

for 10 min. The stained bacteriophage was examined in a Philips EM 300 electron microscope (Philips, Eindhoven, the Netherlands) operated at 80 kV. The phage size was determined from the average of five independent measurements.

Analysis of bacteriophage nucleic acid

Bacteriophage nucleic acid was extracted by using PureLink Viral RNA/DNA Mini Kit (Invitrogen, Carlsbad, CA, USA) according to the protocol provided by the manufacturer. The purified nucleic acid was tested for sensitivity to ribonuclease A, nuclease S_1 and restriction enzyme *Eco*RI (all from Sigma-Aldrich, St. Louis, MO, USA) according to the supplier's recommendations. The results were analyzed by 0.8% agarose gel electrophoresis.

The size of bacteriophage genome was determined by using pulse-field gel electrophoresis (PFGE). The electrophoresis was carried out with 0.8% agarose gel in 0.5x Tris-borate-EDT buffer at 15°C for 15 h, using switch time ramped from 1 to 12 s and a voltage of 6 V/cm. The PFGE size standard used was Low range PFG marker (New England Biolabs, Ipswich, MA, USA).

Table 1. Lytic ability of bacteriophage S14 against different bacterial strains.

Bacteria strain[a]	Bacterial lysis[b]
Aeromonas sobria DMST 1615	-
Aeromonas hydrophila DMST 4997	-
Flavobacterium columnare ATCC 49512	-
Lactococcus garvieae ATCC 43921	-
Plesiomonas shigelloides DMST 3394	-
Pseudomonas fluorescens DMST 0793	-
Streptococcus agalactiae PS01	+
Streptococcus agalactiae ATCC 12403	+
Streptococcus agalactiae DMST 4314	+
Streptococcus agalactiae DMST 4603	+
Streptococcus iniae ATCC 29177	-
Streptococcus iniae ATCC 29178	-
Streptococcus parauberis DSM 6631	-
Vibrio harveyi ATCC 25919	-
Vibrio harveyi ATCC 35084	-
Vibrio vulnificus DMST 1360	-

[a] ATCC = American Type Culture Collection; DMST = Culture Collection for Medical Microorganism Department of Medical Sciences Thailand; DSM = Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
[b] + = inhibition zone formed; - = no inhibition zone formed

RESULTS

When bacteria-free filtrates prepared from 20 tilapia culturing pond water samples were tested for the presence of a bacteriophage specific to *S. agalactiae* PS01 by using spot test method, only one filtrate gave a positive result by causing a clear inhibition zone on the lawn of *S. agalactiae* PS01 (Fig. 1a). By using the agar overlay method, the filtrate also produced clear rounds plaques with the diameter of about 1 mm in diameter on the *S. agalactiae* PS01 lawn (Fig. 1b). These results suggested that the filtrate contain a lytic bacteriophage specific to *S. agalactiae* PS01 that was named as bacteriophage S14.

By using the spot test method to examine the specificity of bacteriophage S14 to a variety of bacteria, it was found that the bacteriophage was virulent against only *S. agalactiae*, but not the rest of bacteria used in this study (Table 1). From

Table 2. Stability of bacteriophage S14 to heat, pH and chemical treatments

Treatment	Bacteriophage survival (%)
Heat treatment	
50°C	95.86
60°C	61.42
70°C	34.57
80°C	0
90°C	0
pH treatment	
pH 2	0
pH 3	32.19
pH 4	49.80
pH 5	85.49
pH 6	90.02
pH 7	96.74
pH 8	95.48
pH 9	90.16
pH 10	88.32
pH 11	47.81
pH 12	0
Chemical treatment	
Malachite green	95.31
Formalin	98.26
Sodium hypochlorite	93.48
50% Benzalkonium chloride	97.53
10% Iodine	99.72
Hydrogen peroxide	97.23
Oxytetracycline	98.44

this result, it was likely that bacteriophage S14 was species specific. It was highly specific to bacteria in the species *S. agalactiae*.

Bacteriophage S14 was stable upon heat treatment at 50°C for 30 min. The temperature dependent decrease of bacteriophage titer was observed as the temperature was increased from 60°C towards 80°C. No bacteriophage S14 was detected at 80°C and 90°C (Table 2).

Bacteriophage S14 was found to be very stable in a wide range of pH. The numbers of surviving bacteriophage maintained above 85% of the initial bacteriophage count in a pH range of 5 to 10. An obvious decrease in bacteriophage titer was observed at pH higher than 10 and lower than 5. AT pH 2 and 12, bacteriophage S14 could not be detected (Table 2).

Sensitivity of bacteriophage S14 to various antimicrobial chemicals is shown in Table 2. All of the chemicals used in this study did not cause significant decrease of bacteriophage titer. The numbers of surviving bacteriophage upon treatments of the chemicals were found to be more than 90% of the initial bacteriophage counts.

Bacteriophage growth cycle parameter including latent period, burst period and burst size were determined from the dynamic change in the number of bacteriophage during one replicative cycle. It was found that bacteriophage PS01 had latent and burst periods of about 40 and 160 min, respectively. The burst size estimated from the one-step growth curve was about 145 PFU/infected cell (Fig. 2).

Transmission electron microscopy revealed that bacteriophage S14 had an icosahedral head of 52 ± 4.6 nm in diameter and a noncontractile tail of 12 ± 0.4 nm in diameter and 210 ± 11.8 nm in length. The bacteriophage did not have other additional structures such as a baseplate, a collar or fibers (Fig. 3).

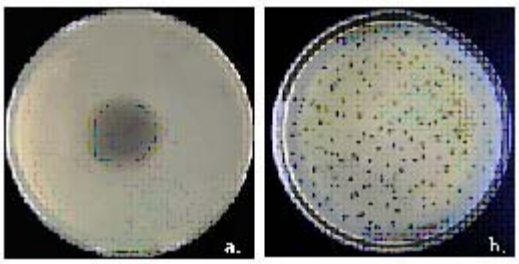


Fig. 1. A clear inhibition zone (a) and plaques on *S. agalactiae* PS01 lawns (b) produced by bacteriophage S14.

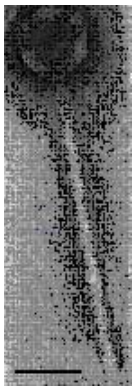


Fig. 3. Transmission electron micrograph of bacteriophage S14. Bar = 50 nm

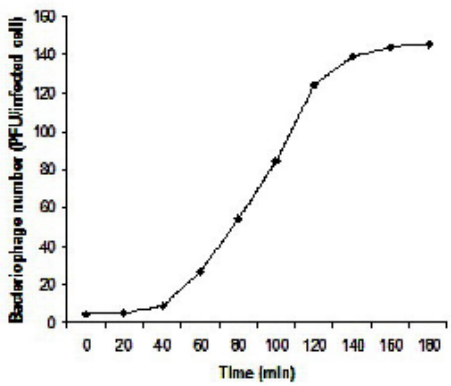
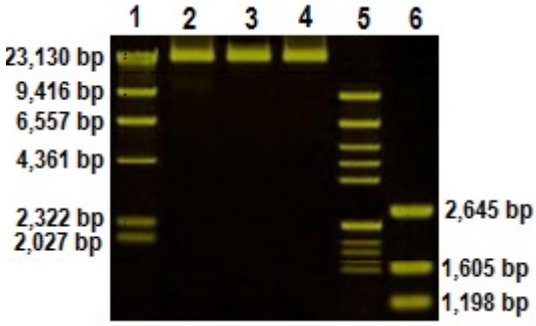


Fig. 2. One-step growth curve of bacteriophage S14



Lane 1, lambda DNA digested with *Hin*dIII marker; Lane 2 uncut of bacteriophage S14 nucleic acid; Lane 3, of bacteriophage S14 nucleic acid digested with ribonuclease A; Lane 4, of bacteriophage S14 nucleic acid digested with nuclease S_1; Lane 5, of bacteriophage S14 nucleic acid digested with *Eco*RI; Lane 6, pGEM DNA marker.

Fig. 4. Agarose gel electrophoresis of bacteriophage S14 nucleic acid.

Results of the digestion of nucleic acid isolated from bacteriophage S14 with different enzymes are shown in Fig. The nucleic acid was tolerant to ribonuclease A and nuclease S_1.while it was cut by *Eco*RI (Fig. 4). The size of the nucleic acid as revealed by the pulsed-filed gel electrophoresis was about 40 kb.

DISCUSSION

Bacteriophage therapy is an approach using a single bacteriophage or bacteriophage combination to prevent and/or treat diseases resulting from bacterial infection. This approach has been considered to be a promising alternative to chemotherapy that depends heavily on antibiotics and has undesired side effects. Bacteriophage therapy has many advantages over chemotherapy. Bacteriophages have high specificity for their target bacteria, indicating that they do not harm the normal intestinal microflora. Bacteriophages are effective against multidrug resistant pathogenic bacteria because the mechanisms by which they induce bacteriolysis differ completely from those antibiotics. Moreover, bacteriophages have self limitation, meaning that the number of phages remain in very low level after killing the target bacteria[21]. Many reports have shown the success of using bacteriophage therapy to control bacterial infectious diseases both in humans and animals[14,15,29,30]. In aquaculture, bacteriophage therapy has gained increasing attention in recent years. Many bacteriophages infecting fish pathogenic bacteria have been discovered and some of them are intensively studied for utilization in fish farms[6,16,19-24].

To use bacteriophage therapy to control bacterial infectious diseases, one of the most important steps needed to be considered is finding a candidate bacteriophage. Although bacteriophages are widespread in environments, they are predominant in places where their hosts occur. Therefore, bacteriophage generally isolated from environments that are habitats for the respective host bacteria. Since in this study *S. agalactiae* used as a main host in bacteriophage screening isolated from tilapia farm environment, tilapia pond water would be ideal for isolation of *S. agalactiae* bacteriophage. Several bacteriophages specific to aquatic animal pathogenic bacteria were isolated from environments where their respective hosts were isolated. Examples of such bacteriophages are those specific to *P. plecoglossicida*[20] and to *V. harveyi*[23,25].

Host specificity of bacteriophage is a significant factor needed to be taken into account. Bacteriophages with very high or very low host specificity can cause problems for being used in bacteriophage therapy. Bacteriophages with a very broad host range spectrum have high tendency to affect beneficial bacteria residing in bacteriophage treated organisms. Use of bacteriophages with narrow host specificity for bacteriophage therapy often causes complexity in preparation of therapeutic bacteriophages. In some cases, combination of several bacteriophages is required for therapeutic application. In this study, bacteriophage S14 was shown to be active against all of the tested *S. agalactiae* strains but not any other tested bacteria, indicating that the bacteriophage had host range spectrum within a species. Bacteriophages with such host range spectrum are desired for bacteriophage therapy because they can be effective against a large number of members of a particular species. Therefore, bacteriophage S14 had proper host specificity for being used as a therapeutic agent to control *S. agalactiae* infectious disease.

Bacteriophage classification requires at least two characteristics of bacteriophage which are type of nucleic acid and morphology. From our study, the nucleic acid of bacteriophage S14 was likely to be double stranded DNA because it was digested by *Eco*RI, a restriction enzyme digesting only double stranded DNA, but not by ribonuclease A, an enzyme digesting RNA, and nuclease S_1, an enzyme active against single stranded DNA and RNA. Transmission electron microscopy revealed that the phage was a tailed phage with an icosahedral head and a long noncontractile tail. Based on these two characteristics, bacteriophage S14 can be classified as a member of the family *Siphoviridae* according to the International Committee on Taxonomy of Viruses[31]. According to the International Committee on Taxonomy of Viruses, bacteriophages with tails or tailed bacteriophages are members of the *Caudovirales* order, an extremely large, morphologically and genetically diverse group that encompasses over 95% of all known

bacteriophages[31]. This order contains three families, namely, the *Myoviridae* (with long, contractile tail), the *Siphoviridae* (with long, noncontractile tail), and the *Podoviridae* (with short tail). Bacteriophages specific to fish pathogenic bacteria have been found in all of these families. For examples, bacteriophages specific to *L. garvieae*[32] and to *F. psychrophlum*[18] were placed in the family *Siphoviridae* while bacteriophages specific to *P. plecoglossicida* were found to be members of the family *Myoviridae* and *Podoviridae*[20,33].

Sensitivity of bacteriophage to pH, temperature and biocides is an important key to success for using bacteriophages as therapeutic agents. This study showed that bacteriophage S14 was tolerant to relatively high temperature ranging from 50°C to 70°C and to a broad range of pH. These characteristics may allow the bacteriophage to be used in broad application and in the environments having fluctuated temperature and pH. Our study also showed that bacteriophage S14 was stable in the presence of biocides commonly used in aquaculture including malachite green, formalin, sodium hypochlorite, 50% Benzalkonium chloride, 10% iodine, hydrogen peroxide and oxytetracycline. According to the results, it is possible to use bacteriophage S14 in fish culturing ponds having residues of the compounds. Furthermore, it may be used in combination with the compounds to improve its therapeutic efficacy or to reduce the amount of the compounds being applied.

In conclusion, our study clearly indicates that the bacteriopahge S14 is a lytic bacteriophage specific to bacteria in the species of *S. agalactiae* and stable to high temperature, a wide range of pH and several antimicrobial compounds. Although these characteristics favor its use for prophylactic and therapeutic purposes in aquaculture, further investigations are still required before using bacteriophage S14 to control *S. agalactiae* infectious diseases in an actual situation. Research works that is now going on in our laboratory designed to elucidate the following issues: (1) the survival, infectivity and effect on bacterial community of the bacteriophage in fish and in culturing environments (2) the effect of the bacteriophage on growth, immunity and behavior of fish and (3) the most effective way to deliver the bacteriophage into fish body. Information from these works may be useful for application of bacteriophage S14 as therapeutic agents to control *S. agalactiae* infection in fish.

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