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Short communication

A lytic bacteriophage of the newly emerging rainbow trout pathogen *Weissella ceti*

Truong Dinh Hoai[a,c], Kyoka Mitomi[a], Issei Nishiki[b,*], Terutoyo Yoshida[a]

[a] Faculty of Agriculture, University of Miyazaki, Gakuen Kibanadai Nishi 1-1, Miyazaki 889-2192, Japan
[b] Research Center for Bioinformatics and Biosciences, National Research Institute of Fisheries Science, Japan Fisheries Research and Education Agency, 2-14-4 Fukuura, Kanazawa, Yokohama 236-8648, Japan
[c] Faculty of Fisheries, Vietnam National University of Agriculture, Hanoi, Viet Nam

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ABSTRACT

This study was conducted to isolate and characterize a bacteriophage of a newly emerging pathogen, *Weissella ceti*, which causes weissellosis outbreaks of intensively farmed rainbow trout worldwide. The phage appeared together with the cultured *Weissella ceti* during isolation of pathogen from kidney of diseased rainbow trout. The morphological, physiological, proteomic and lytic spectrum were characterized. This phage, named PWc, belonged to the family *Siphoviridae* and possessed an isometric head (approximately 65 nm in diameter) and a flexible, non-contractile tail of 170–180 nm in length. The latent time and burst size of PWc were approximately 25 min and 16 PFU/infected cells, respectively. The PWc was relatively stable over a wide range of temperatures and pH values and possessed a broad lytic spectrum, lysing all 36 tested *W. ceti* strains isolated from diseased rainbow trout in Japan. The protein profile of the phage was obtained using SDS-PAGE analysis, and the potential packaging strategy was determined based on terminase large subunit sequence analysis. This is the first study to investigate a lytic bacteriophage of a newly emerging pathogen *W. ceti* that causes infectious disease in rainbow trout.

The genus *Weissella* comprises Gram-positive, catalase-negative, non-endospore–forming coccoid or rod-shaped cells; and contains 23 recently investigated species that belong to the group of bacteria known as lactic acid bacteria (Björkroth et al., 2014; Fusco et al., 2015; https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=46255). These bacterial species have been detected in a wide range of nutrient-rich habitats such as skin, milk, animal and human feces, plants, vegetables, and a variety of fermented foods (Choi et al., 2002; Nistal et al., 2012; Patel et al., 2013). Several species in this genus have been considered to exhibit high probiotic potential for controlling periodontal diseases as prebiotics and have a broad range of industrial applications such as in the production of flour-based baked food and the production of cereal-based fermented functional beverages (Fusco et al., 2015). However, several species of this genus are known to be opportunistic pathogens involved in human infections (Lee et al., 2011). Therefore, bacteria belonging to this genus are important in both biotechnological and medical aspects. Several Weissella phages have been investigated recently, and almost all classified phages were specific to *Weissella cibaria* isolated from fermented food, such as phage Φ22 attacking *W. cibaria* from Thai fermented pork sausage (Pringsulaka et al., 2011), phages Φ 3.8.18, Φ 7.2.50, and Φ 3.8.43 attacking *W. cibaria* from cucumber fermentation (Lu et al., 2012; Kot et al., 2014), and Φ YS61 attacking *W. cibaria* from kimchi fermentation (Kleppen et al., 2012).

Weissellosis is a rapidly emerging disease of the intensively farmed rainbow trout (*Oncorhynchus mykiss*), with recent outbreaks in China (Liu et al., 2009), Brazil (Costa et al., 2015; Figueiredo et al., 2012), the United States (Welch and Good, 2013) and Japan (Mitomi et al., 2018). The partial comparison of 16S rRNA sequences obtained from these outbreaks and the rapid identification and quantification of isolates indicated that the worldwide outbreaks in trout were caused by the same novel species, *Weissella ceti* (Snyder et al., 2015). The outbreak of *W. ceti* infection resulted in the extensive and prolonged mortality of market-sized fish and significant economic loss (Welch and Good, 2013). Rainbow trout is an important species and contributes significantly to the global aquaculture production, and weissellosis is predicted to become a major problem for the aquaculture industry in the future (Welch et al., 2017). Therefore, developing a method to prevent this disease appropriately is urgently required. In the present study, a lytic *W. ceti* bacteriophage with the potential to be used as a therapeutic agent against weissellosis in rainbow trout was isolated and characterized.

* Corresponding author.
E-mail address: nishikii@affrc.go.jp (I. Nishiki).

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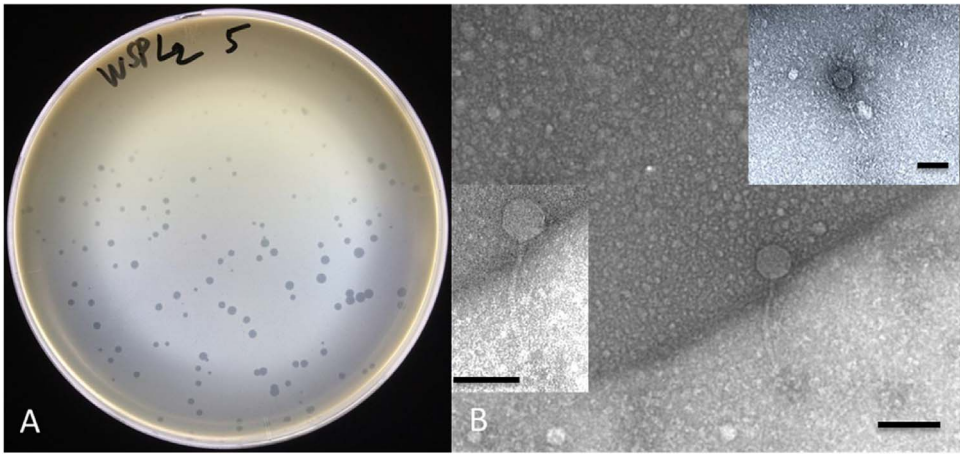

Fig. 1. Plaques and morphology of phage PWc. Bar, 100 nm.

During investigation of diseased rainbow trout, phage plaques were detected on cultured bacteria *Weissella ceti* isolated from the kidney of diseased rainbow trout. Phage plaques were then collected, maintained in SM buffer (100 mM/L NaCl; 7 mM/L $MgSO_4{\cdot}7H_2O$; 50 mM/L Tris-HCl, pH 7.5; and 0.01% w/v gelatin), and incubated at room temperature for 2 h and then at 4 °C overnight. The solution was centrifuged (at 10,000*g* for 3 min at 4 °C) and filtered through a 0.45 μm cellulose acetate membrane. The presence of the phage was confirmed again by performing a spotting test for the bacterial lawn of the original *W. ceti* strain (MW1608-01) wherein the phage was detected. The phage was isolated by the plaque assay using the double layer agar method (Adams, 1959). An individual plaque was harvested and suspended in SM buffer and then re-isolated three times to ensure purity of the isolated phage. The phage was isolated and clear plaques, with diameters of 0.7–2 mm (Fig. 1A), were obtained from the bacterial lawn. The phage isolate was successfully purified by performing the double layer agar method three times, the purified stock of the phage was prepared by the plate lysis method stored at 4 °C for further use (Sambrook et al., 1989). To determine the morphological features of the phage, phage sample was examined under a JEM-2010 MX biological transmission electron microscope (JEOL, Japan) operated at an accelerating voltage of 100 kV. The phage size was determined from the average of five independent measurements. TEM analysis revealed that PWc possessed an isometric head approximately 60–65 nm in diameter and a long, flexible, and non-contractile tail approximately 170–180 nm in length and 9–10 nm width (Fig. 1B). According to the morphological features and based on the classification of Ackermann (2001), PWc was revealed to belong to the family *Siphoviridae*.

To determine the lytic spectrum of the phage, a collection of bacterial strains, including 36 *W. ceti* strains isolated from diseased rainbow trout in South Japan from 2012 to 2016 confirmed by the duplex PCR (Snyder et al. (2015); Fig. S1), a *W. ceti* strain 1119-1A-09 isolated from beaked whale, purchased from the Spanish type culture collection (Vela et al., 2011), two *Weissella cibaria* strains and a lactic acid bacterial strain, were used to test for sensitivity against PWc (Table 1). Bacteria were preserved at −80 °C. A liquid culture medium and a solid medium (Bacto Todd Hewitt Broth [THB] and THB with agar [THA], respectively; Difco, Sparks, MD, USA) supplemented with 5% sheep blood (THA-blood) were used to aerobically culture each bacterial strain at 25 °C. The phage host range was determined by the spot assay. In brief, bacterial lawns were formed by adding 100 μL of exponential phase cell culture of each isolate to be tested to 3 mL of molten top soft agar THA-blood (0.7% agar) and overlaid on the surface of solidified base THA (1.5% agar). Subsequently, 5 μL of the phage suspension (10^8 PFU/mL) was spotted on the plate. The plates were incubated at 25 °C overnight, and phage susceptibility was evaluated by either a uniform clear plaque formation or the appearance of individual plaques at the phage spot site. The results of the test showed that PWc possessed a broad lytic spectrum and infected all the tested *W. ceti* strains (n = 36) isolated from diseased rainbow trout in Japan. PWc could not lyse *W. ceti* strain 1119-1A-09 (beaked whale strain), *W. cibaria* strains, and *Lactococcus lactis* subsp. *lactis* (ATCC19435) (Table 1).

To obtain the latent period and burst sizes of the phage, exponential bacterial cultures of the host bacterial strain was adjusted to an OD of 0.8 at 600 nm (equivalent to a cell density of 10^8 CFU/mL) in 15 mL fresh THB. Phage suspension was added with a multiplicity of infection (MOI) of approximately 1.0. The phage was allowed to adsorb at 25 °C for 5 min. The mixture was centrifuged at 12,000*g* for 1 min at 4 °C to remove unadsorbed phage particles. The resultant pellet was re-suspended in 15 mL of THB and incubated at 25 °C. One mL of the suspension was collected in 5 min intervals and centrifuged immediately for 30 s at 4 °C after collection for phage counts using the double-layer agar assay. The plates were incubated at 25 °C and examined for plaques after 12–16 h. The experiment was independently performed three times. The latent periods and burst sizes were calculated from one-step growth curves. As shown in Fig. 2, the latent time, rise, and plateau phases were obtained, in which the latent time and burst size of PWc were determined to be about 25 min and 16 PFU/infected cell, respectively.

To determine the phage resistance against different pH values, pH of the THB broth was adjusted to a range of 1–10 using NaOH and HCl (Wako, Japan). In brief, 100 μL of the phage suspension (2×10^{11} PFU/mL) was inoculated into 9.9 mL of each pH-adjusted medium (final phage concentration of approximately 2×10^9 PFU/mL). After incubation for 1 h at 25 °C, the active phage rate was determined by counting phage viability under specific pH conditions using the double layer agar assay. To examine the thermostability of the phage, the phage suspension was incubated at different temperatures of 40, 50, 60, 70, 80, and 90 °C for 1 h. The survival rate of each treated phage sample was determined at 15 min intervals using the double-layer agar assay. The plates were incubated at 25 °C overnight to evaluate plaque development. All assays were performed three times. The optimal pH of PWc was determined by testing its stability within 1 h under pH values ranging from 1 to 10. The highest stability of PWc was obtained at around pH 7.0. PWc was stable for a wide range of pH values, with more than 60% active phage rate at pH 4–10 and more than 40% at pH 3.0. A few phage plaques were observed at pH 2.0. However, the phage completely lost its infectivity at pH 1.0 (Fig. 3). The results of the thermal stability test shown in Fig. 4 suggested that PWc was relatively stable over 1 h between 40 and 60 °C with more than 90% survival at 40–50 °C and 63.3% at 60 °C. The phage titer quickly decreased by 90% after 15 min, and no phage particles were detected after 30 min at 70 °C. At 80 and 90 °C, no phage particles were detected after 15 min.

The purified phage suspension at a high concentration (approximately 10^{10} PFU/mL) was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (Ghasemi et al., 2014)