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NOTE

A virulent bacteriophage of *Lactococcus garvieae* (formerly *Enterococcus seriolicida*) isolated from yellowtail *Seriola quinqueradiata*

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ABSTRACT: A virulent bacteriophage, designated PLgY, was detected from cultures of *Lactococcus garvieae* (formerly *Enterococcus seriolicida*) isolated from diseased yellowtail *Seriola quinqueradiata*. The phage had an isometric head measuring 50 to 60 nm, a thin flexible tail of 7 × 140–180 nm, and the genome consisting of double stranded DNA, indicating that PLgY is a member of the family Siphoviridae. Of 26 strains of *L. garvieae* examined, 24 were sensitive to the phage but 2 strains of *L. garvieae* and another 22 strains of bacteria including fish and shellfish-pathogenic bacteria were not. Lysis of *L. garvieae* cells due to the phage infection was dependent on culture temperature and occurred at between 17 and 29°C. Although an infection experiment of young yellowtail revealed that the 2 phage-insensitive *L. garvieae* strains were less virulent than 2 phage-sensitive strains, there was no correlation between phage sensitivity and antigenic variation.

KEY WORDS: Bacteriophage · Siphoviridae · *Lactococcus garvieae* · *Enterococcus seriolicida* · Yellowtail · *Seriola quinqueradiata*

The disease caused by *Enterococcus seriolicida* (Kusuda et al. 1991) is one of the most important diseases of cultured yellowtail *Seriola quinqueradiata* in Japan, and has long been known as streptococcal infection because of the name, *Streptococcus* sp. (Kusuda et al. 1976), originally given to the causative agent. The most recent studies on the taxonomic position of *E. seriolicida* based on DNA homology have revealed that this is a junior synonym of *Lactococcus garvieae* (Teixeira et al. 1996, Eldar et al. 1996). The name *L. garvieae* instead of *E. seriolicida* is therefore used in this paper.

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The disease prevails in the summer when the water temperature rises above 20°C although the pathogen can be detected from yellowtail culture environments (sea water and sediments) throughout the year (Kitao et al. 1979). Reducing stress factors, such as poor water quality and insufficient nutrition, is generally known to be important in controlling enterococcal infection (Plumb 1994), and a killed bacterin has proved to be effective in preventing *Lactococcus garvieae* infection of yellowtail in experimental conditions (Iida et al. 1982). However, the infection mechanism of the disease is not yet fully understood.

Plaques which were thought to be formed by bacteriophages were observed on colonies of *Lactococcus garvieae* isolated from diseased yellowtail during routine diagnostic work in Ehime Prefecture, Japan. In this paper we report isolation, growth conditions, and characteristics of a bacteriophage isolated from *L. garvieae*.

Materials and methods. ***Bacteria and media:*** Twenty-five strains of *Lactococcus garvieae*, which were isolated from the kidney of diseased yellowtail at different farms in Ehime Prefecture, were used in this study. *Enterococcus seriolicida* YT-3 (= ATCC49156^T), which was provided by Dr R. Kusuda, was used as a reference strain. Anti-*L. garvieae* rabbit sera raised against 2 strains belonging to different antigenic phenotypes (KG– and KG+; Kitao 1982), which were provided by Dr T. Yoshida, were employed to type the *L. garvieae* strains used. Another 22 species including fish- and shellfish-pathogenic bacteria were used to determine the host range of the phage. These were *Aeromonas hydrophila* ET-2, *A. salmonicida* NCMB1102, *Edwardsiella tarda* NUF251, *Enterococcus faecalis* JCM5803, *Ent. faecium* JCM5804, *Escherichia coli* WP2, *Photobacterium damsela piscicida* (= *Pasteurella piscicida*)

SP91142, *Pseudomonas anguilliseptica* NCMB1949, *Ps. fluorescens* 03L, *Streptococcus equisimilis* HUFP9503, *S. iniae* HUFP9502, *Vibrio alginolyticus* HUFP9107, *V. anguillarum* (= *Listonella anguillarum*) HUFP5001, non-O1 *V. cholerae* PS7702, *Photobacterium damsela* (= *V. damsela*) ATCC33539, *V. harveyi* HUFP9111, *V. ichthyoenteri* F-2, *V. ordalii* ATCC-33509, *V. penaeicida* KH-1, *V. parahaemolyticus* HUFP-9004, *V. salmonicida* ATCC43939, and *V. vulnificus* ES-7601. Trypto-soya broth (TSB, Nissui) and Trypto-soya agar (TSA, Nissui) were used for bacterial culture and the plaque forming unit (PFU) assay of phage.

Isolation of phage and PFU assay: Phages were isolated by using a double agar layer method (Paterson et al. 1969). A small amount of *Lactococcus garvieae* colonies with plaques was removed from the plate and suspended in TSB. The suspension, mixed with soft TSA (0.35% agar, kept at 50°C), was poured onto a TSA plate and incubated at 25°C for 24 h. The layer of soft agar showing plaques was removed from the plate and centrifuged at 12 000 × *g* for 5 min. The supernatant was filtered through a 0.45 µm membrane filter and stored at 4°C until required. By this procedure, 4 phage isolates, designated as PLgY nos. 4, 9, 16, and 22 (PLgY = phage of *Lactococcus garvieae* from yellowtail) were obtained from their initial host strains. Prior to experiments, these phages were propagated with each initial host strain by the method described above and the phage concentration was adjusted to between 10^8 and 10^9 PFU ml^{-1}. To determine the PFU of phage 0.1 ml of serial 10-fold dilutions of the phage suspension was mixed with a 0.4 ml bacterial suspension (optical density, OD = 0.05), and then put into 3 ml of soft TSA (50°C), poured onto a TSA plate, and incubated at 25°C for 24 h.

Growth characteristics of phage: PLgY no. 16 and *Lactococcus garvieae* strain no. 16 were used in the following experiments. A 5 ml bacterial suspension (OD = 0.05) was mixed with 0.2 ml of phage suspension (10^3 PFU ml^{-1}) and kept at 25°C for 10 min. Unabsorbed phages were removed by 3 washings with centrifugation at 12 000 × *g* for 5 min at 4°C, and then 0.1 ml of bacteria infected with phage was incubated in another 5 ml of the bacterial suspension (OD = 0.05). During 7 h incubation with shaking at 25°C, OD and PFU were measured every hour to estimate bacterial and phage growth, respectively. In another experiment, a mixture of 0.2 ml of a phage suspension (10^3 PFU ml^{-1}) and 10 ml of a bacterial suspension (OD = 0.02) was incubated at different temperatures ranging from 10 to 48°C by a temperature gradient shaking incubator. After an 8 h incubation, the OD was read in order to demonstrate bacterial cell lysis.

Electron microscopy of phage: Phage suspensions (10^8 to 10^9 PFU ml^{-1}) were placed on grids and negatively stained with 5% phosphotungstic acid, and then examined with an electron microscope (Hitachi-H600) at 80 kV.

Analysis of the phage nucleic acids: Nucleic acids of phages were extracted with phenol-saturated TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) followed by a mixture of chloroform and isoamyl alcohol (24:1). The nucleic acids were precipitated by the addition of a 0.1 volume of 3 M ammonium acetate and a 2.5 volume of absolute ethanol, dried, and resuspended in diethyl pyrocarbonate-treated water. The purified nucleic acids were tested for sensitivities against RNase (20 µg ml^{-1}), DNase (20 µg ml^{-1}), and the restriction enzyme *Eco*RI (20 U ml^{-1}) after incubation in 50 mM Tris-HCl buffer (pH 7.5) at 37°C for 2 h. The results were examined by 1% agarose gel electrophoresis at 100 V for 30 min.

Pathogenicity of Lactococcus garvieae strains: Two phage-sensitive strains (nos. 16 and 22) and 2 phage-insensitive strains (nos. 1 and 11) of *L. garvieae* were used to compare their pathogenicity to young yellowtail weighing 30 to 40 g. Groups of 10 fish were injected intraperitoneally with bacteria at doses of 10^6 and 10^7 colony forming units (CFU) per fish. The inoculated fish were then kept in 40 l plastic tanks with flow-through water at 23 to 25°C. Mortalities were recorded daily for 3 wk, and kidneys of dead fish were submitted to bacterial isolation.

Results and discussion. An electron micrograph of negatively stained PLgY no. 16 phage is shown in Fig. 1 This and another 3 phages (PLgY nos. 4, 9, 22) had an isometric head measuring 50 to 60 nm and a thin flexible tail of 7 × 140–180 nm. Fig. 2 shows the nucleic acid analysis of PLgY no. 16 phage indicating that the nucleic acid was larger than 20 kbp. In addition it was broken down by DNase and *Eco*RI, but not by RNase. The same results were obtained with the other PLgY phages. These morphological and genomic characteristics indicate that the present PLgY phage can be classified into the family Siphoviridae by current taxonomy (Murphy et al. 1995).

Twenty-four strains, out of 26 *Lactococcus garvieae* strains including the reference strain YT-3, were sensitive to 4 PLgY phages with PFUs ranging from $10^{7.3}$ to $10^{9.7}$ ml^{-1}, but the remaining 2 strains (nos. 1 and 11) were not (Table 1). After 24 h incubation plaques were round in shape and ranged from 0.3 to 3 mm in diameter. The size of plaques was dependent on the bacterial strains but not on the phage isolates. As reported by Kitao (1982), *Enterococcus seriolicida* (= *L. garvieae*) from diseased yellowtail was divided into 2 antigenic types; KG– and KG+. Strains on initial isolation always belong to the KG– type but surface antigen(s) of KG– cells are lost during subculture, and strains change to the KG+ type. The KG– type is char-

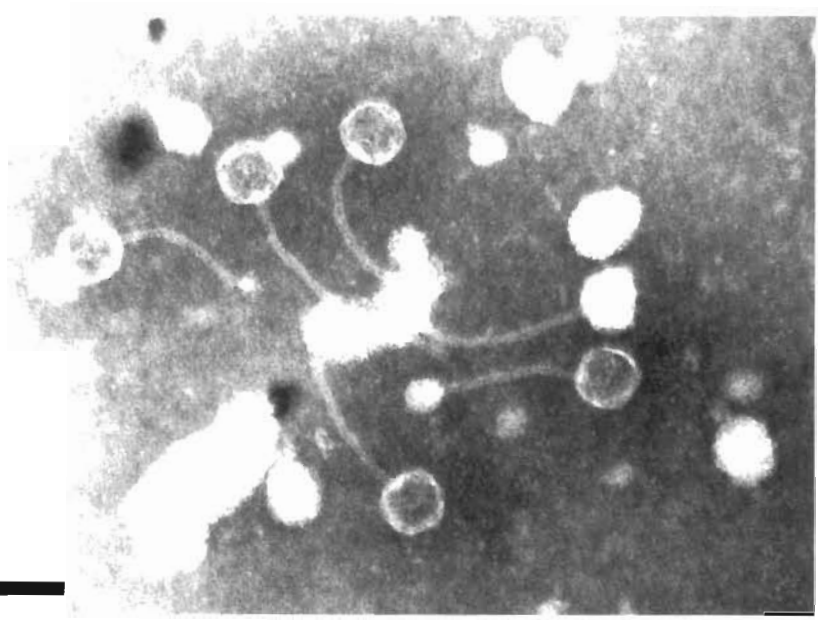

Fig. 1. Electron micrograph of negatively stained PLgY phage no. 16. Scale bar = 100 nm

acterized by its inability to agglutinate to an antiserum against KG+ cells. All *L. garvieae* strains used in the present study were positive to the anti-KG– serum but there were no correlations between the phage sensitivity and the surface antigenicity. It has also been shown that antigenic conversion from KG– to KG+ is followed by decrease of virulence to fish (Alim et al. 1996) and that KG– cells are more hydrophilic than KG+ cells and are resistant to phagocytosis by yellowtail kidney phagocytes (Yoshida et al. 1996). In the present study, we examined the pathogenicity of 4 selected *L. garvieae* strains to yellowtail and found that 2 phage-sensitive strains (nos. 16 and 22) were more virulent than phage-insensitive strains (nos. 1 and 11) (Table 2). However, the positive relationship between antigenic type and virulence was not observed in our experiment because strains nos. 11, 16 and 22 were type KG– and strain no. 1 was type KG+.

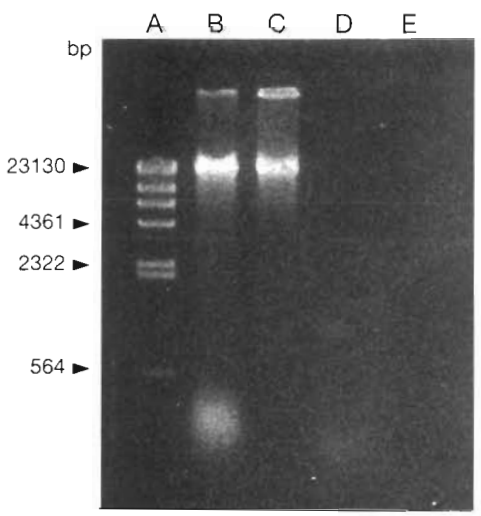

Fig. 2. Nucleic acid analysis of PLgY no. 16 phage by agarose-gel electrophoresis. A: marker (λ/*Hind*III digest), B: intact, C: treated with RNase, D: treated with DNase, E: treated with *Eco*RI

Table 1 Antigenic types of *Lactococcus garvieae* strains and sensitivities of the strains to 4 PLgY phage isolates (nos. 4, 9, 16, 22)

L. garvieae strain no.	Antigenic type	Range of phage PFU (log_{10} ml^{-1})
1	KG+	<1.0
2	KG–	8.3–9.4
3	KG–	8.0–9.7
4	KG+	8.3–9.3
5	KG–	8.6–9.5
6	KG–	8.2–9.7
7	KG–	8.3–9.6
8	KG–	8.0–9.1
9	KG+	7.9–9.0
10	KG+	8.3–9.1
11	KG–	<1.0
12	KG+	8.4–9.3
13	KG–	7.4–9.3
14	KG+	8.1–9.4
15	KG–	8.3–8.8
16	KG–	8.3–9.3
17	KG–	7.9–8.7
18	KG–	8.3–9.1
19	KG–	8.3–9.3
20	KG–	8.4–9.1
21	KG–	8.1–8.4
22	KG–	7.6–9.1
23	KG–	7.7–8.8
24	KG–	7.7–8.6
25	KG–	7.3–8.7
YT–3	KG+	7.3–8.1

Table 2. Pathogenicity of selected *Lactococcus garvieae* strains to yellowtail *Seriola quinqueradiata*. Fish (30 to 40 g in body weight; N = 10) were injected intraperitoneally with strains and observed at 23 to 25°C for 3 wk

L. garvieae strain no.	Dose injected (CFU fish^{-1})	Mortality (%)
1	7.0×10^7	0
	7.0×10^6	0
11	7.0×10^7	0
	7.0×10^6	0
16	1.2×10^7	60
	1.2×10^6	20
22	3.0×10^7	60
	3.0×10^6	10

The other 22 bacterial strains tested, including fish and shellfish pathogens, were not sensitive to the phage, indicating that the PLgY phage was specific for *Lactococcus garvieae* and potentially applicable for disease diagnosis, at least in yellowtail. Phage typing is a well known sensitive tool for establishing generic relationships among intra-species of bacteria (Pfaller 1991). A comprehensive phage typing scheme for the fish pathogen *Aeromonas salmonicida* was described where 27 phage groups of *A. salmonicida* were distinguished based on the sensitivity patterns to 18 phage isolates (Rodgers et al. 1981). Further work with more *L. garvieae* strains and phage isolates from different sources would be required in order to construct such a phage typing scheme for this pathogen.

There were no differences in the growth of *Lactococcus garvieae* strain no. 16 between phage-free and phage-infected cultures in the first 3 h of incubation, but after 4 h a clear difference was observed (Fig. 3). The growth of phage-infected bacteria slowed down after 4 h and cell density showed a sudden reduction

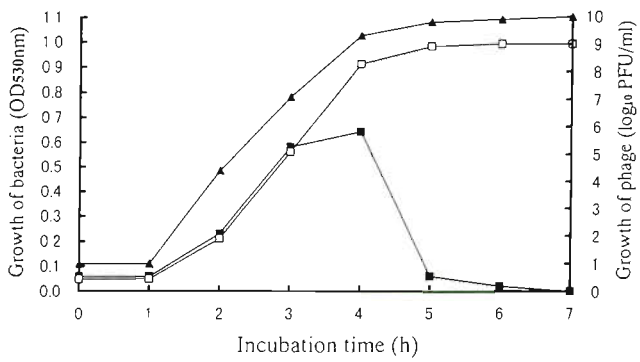

Fig. 3. Growth of *Lactococcus garvieae* infected with PLgY phage and extracellular phage titers. *L. garvieae* strain no. 16 infected with PLgY phage no. 16 was cultured by shaking at 25°C. (■) OD of bacteria infected with phage, (□) OD of bacteria uninfected with phage, (▲) PFU of phage

Table 3. Effect of incubation temperature on the lytic activity of the phage PLgY no. 16. *Lactococcus garvieae* strain no. 16 was cultured with or without the phage at different temperatures. na: not applicable

Incubation temperature (°C)	Growth of bacteria (without phage)	Lysis of bacteria (with phage)
10–14	–	na
17–29	+	+
32–41	+	–
44–48	–	na

after 5 h. Extracellular phages appeared after a latent period of 1 h and then progeny increased exponentially until reaching the maximum number of 10^{10} PFU ml^{-1} after 5 h. Table 3 shows the effect of incubation temperature on the lytic activity of phage PLgY no. 16. Bacteria uninfected with phage grew well at 17 to 41°C, but lytic activity of the phage was observed only at temperatures between 17 and 29°C. *In vivo* relationship between PLgY phage and *L. garvieae* will be investigated in a future study.

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