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New Temperate Bacteriophages of *Lactococcus garvieae*

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Temperate phages were effectively induced from presumptive lysogenized cells of 96 strains out of 111 strains of *L. garvieae* No. 44 strains (phage type B) as the host cell. Similar cultures in distilled water-based TSB did not induce lytic infection in these cells. These temperate phages were also effectively induced by ultraviolet irradiation. All phages isolated were lytic only to *L. garvieae* No. 44 strain and the lytic nature was different from those of PLgY, PLgW, and PLgS. The virions appeared extracellularly after 1h of induction culture and increased in number until reaching the maximum of 10^6 PFU/ml after 12h. This phage production was lower than that (10^{10} PFU/ml) of the virulent phage.

Key words: *Lactococcus garvieae*, Temperate bacteriophage, Lysogeny, Induction of phage, Lytic infection

Usually the presence of the phage becomes noticeable by development of plaque on the bacterial colonies. However, such a lytic cycle sometimes does not occur. Instead, the phage DNA becomes incorporated as a prophage into the host's DNA, a state called lysogeny. The lysogenized bacterial cell may exhibit new properties such as toxin production. For example, diphtheria toxin is formed only by strains of *Corynebacteriam diphtheriae* that are lysogenized by phage β, and its production is coded for by genetic information present in the phage genome. Nontoxigenic and hence nonpathogenic *C. diphteriae* can be converted to pathogenic strains by infection with the phage (Brock and Madigan, 1991). Therefore, the investigation on the presence of temperate phages in *Lactococcus garvieae* seems important.

The disease caused by *Enterococcus seriolicida* (Kusuda *et al.*, 1991) is one of the most important diseases of cultured yellowtail Seriola quinqueradiata in Japan. The causative agent of this disease, which has long been called streptococcal infection, was first reported as *Streptococcus* sp. (Kusuda *et al.*, 1976) and identified as *Enterococcus seriolicida* n. sp. in 1991. However, it was recently demonstrated that *E. seriolicida* is a junior synonym of *Lactococcus garvieae* based on phenotypic and genotypic homologies (Teixeira *et al.*, 1996; Eldar *et al.*, 1996). The name *L. garvieae* instead of *E. seriolicida* is therefore used in this paper.

In a previous study (Park *et al.*, 1997; Park *et al.*, 1998), we found a virulent bacteriophages specific to *L. garvieae* isolated from diseased yellowtail, sea water and sediment samples. Also, one hundred and eleven strains of *L. garvieae* examined were divided into 14 phage types (A-N) by using the phage isolates which were differentiated from each other in the infectivity.

After lytic infection of a virulent *L. garvieae* phage PLgY16, a small number of bacterial colonies of *L. garvieae* strain No. 16 appeared on agar plate. The

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cells derived from these surviving colonies were insensitive to the PLgY16 phage even after 20 passages, suggesting a possibility that this insensitivity was due to lysogenic infection with some temperate phages. In the present study, we attempted to detect temperate phages of *L. garvieae*.

Materials and Methods

Bacteria

One hundred and eleven strains of Lactococcus garvieae including two reference strains, *L. garvieae* ATCC 43921T and *Enterococcus seriolicida* ATCC 49156T, were used in this study. They included 93 strains from yellowtail, 6 strains from purplish amgerjack *Seriola dumerili*, 3 strains form Japanese flounder *Paralichthys olivaceus*, 1 strain from gold-striped amberjack *S. laladi*, and 3 strains from sea water. Their geographical sources are shown in Table 1. These strains were provided by some Prefectural Fish Disease Control Centers or Fisheries Research Stations in Japan and stored in tryptic soy broth (TSB; Nissui) with 10% glyserol at -80°C and subcultured on tryptic soy agar (TSA; Nissui) at 25°C for 48h prior to the onset of experiments. These strains were confirmed to be *L. garvieae* based on their morphological and major biochemical characteristics. Anti-*L. garvieae* rabbit sera raised against two strains belonging to different antigenic types (KG- and KG+) (Kitao, 1982) were employed in typing *L. garvieae* strains.

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

Source	Number of strains
Ehime Prefecture, Japan, 1994-1996	47
Kagoshima Pref., Japan, 1996	19
Nagasaki Pref., Japan, 1994-1996	9
Mie Pref., Japan, 1993-1996	8
Oita Pref., Japan, 1996	10
Wakayama Pref., Japan, 1995-1996	15
Miyazaki Pref., Japan, 1993	1
Reference strain	*E. seriolicida* ATCC49156^T
Reference strain	*L. garvieae* ATCC43921^T

Induction of temperate phage

A small amount (10^3 CFU) of each *L. garvieae* strain was inoculated in TSB prepared with sea water and incubated at 25°C for 24h. The sea water was collected at Fisheries Laboratory of Hiroshima University (Takehara) in Japan and autoclaved. Sometime an artificial sea water (Sen Ju Co., Ltd., Osaka, Japan) was used. After centrifugation at 12,000×g for 10 min, the supernatant was tested for the presence of phages by the double agar overlay method. 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). 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 24h. Represen-

Table 2. Sources of the *Lactococcus garvieae* strains used as the host cell in the temperate phage induction test

Phage type	*L. garvieae strains*	Source
A	Nos. 2, 4, 6, 8, 9, 10, 12, 14, 16, 18, 20, 22, 23, 24	Ehime Prefecture, Japan, 1994
	Nos. 39, 40, 41, 42, 45, 46, 47	Ehime Pref., Japan, 1996
	E. seriolicida ATCC49156^T	Reference strain
B	No. 44	Ehime Pref., 1996
C	No. 53	Kagoshima Pref., 1996
D	No. 81	Mie Pref., 1996
E	Nos. 51, 52	Kagoshima Pref., 1996
F	No. 112	Wakayama Pref., 1996
G	Nos. 107, 113	Wakayama Pref., 1996
H	No. 94	Wakayama Pref., 1996
I	No. 64	Oita Pref., 1996
J	No. 38	Ehime Pref., 1996
	Nos. 55, 60, 61	Kagoshima Pref., 1996
	No. 108	Wakayama Pref., 1996
K	No. 67	Kagoshima Pref., 1996
L	No. 96	Oita Pref., 1996
M	No. 95	Oita Pref., 1996
N	Nos. 1, 11	Ehime Pref., 1994
	No. 99	Oita Pref., 1996

tative *L. garvieae* strains of each phage type (A-N) were used as host cells to detect phages (Table 2).

Method for ultraviolet irradiation

A small amount (10^3 CFU) of each *L. garvieae* strain was suspended in TSB and poured in autoclaved petri-dish. This bacterial suspension was irradiated with ultraviolet at 31 μW/cm² for 10, 30, and 60 min and inoculated in TSB prepated with sea water and incubated at 25°C for 24h.

Growth characteristics of phage

The lysogenized *L. garvieae* No. 16 strains was cultured in TSB which was prepared with sea water or distilled water. During 12h incubation with shaking at 25°C, OD and PFU were measured every hour to estimate bacterial and phage growths, respectively.

Results

Phages were obtained from 96 strains of 111 *L. garvieae* strains examined when *L. garvieae* No. 44 strain (phage type : B) was used as the host cell. These temperate phages were also effectively induced by ultraviolet irradiation at 31 μW/cm² for 10-60 min. In cases that other phage type strains were used as host cells in natural and artificial sea water-TSB or distilled water-based TSB for the phage induction, no plaques appeared on their double layered agar plates.

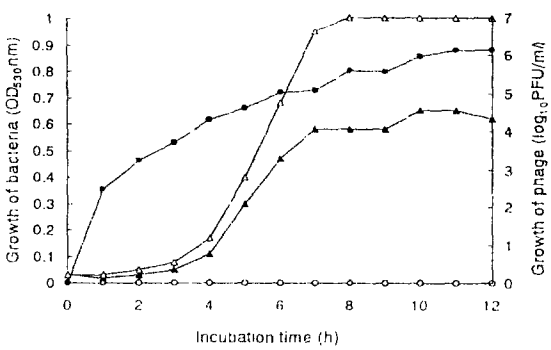

Fig. 1. Growth of *Lactococcus garvieae* No. 16 and induction of the temperate phage from the culture of *L. garvieae* No. 16 strain. (▲) OD_{530nm} of bacteria in sea water-TSB, (△) OD_{530nm} of bacteria in distilled water-TSB, (●) PFU of phage in sea water-TSB, (○) PFU of phage in distilled water-TSB.

As shown in Fig. 1, the growth of *L. garvieae* No. 16 was observed until reaching the maximum density of OD_{530nm} 1.0 in distilled water-based TSB but the maximum density of the bacterium induced for temperate phage was OD_{530nm} 0.6 in sea water-TSB. The temperate phage appeared extracellularly after 1h culture and increased until reaching the maximum number of 10^6 PFU/ml after 12h. However, no phage production was observed in the bacterium cultured in distilled water-based TSB.

Discussion

A large number of different bacterial species are known to harbor temperate phages. Temperate phages have sometimes been discovered by the same method as virulent phages by testing cultures for plaque-forming virus, which is nearly always present at low levels in cultures of lysogens (= lysogenized cells).

Phages were obtained from 96 strains of 111 *L. garvieae* strains examined when *L. garvieae* No. 44 strain (phage type : B) was used as the host cell. All phages isolated in the present study were lytic only to *L. garvieae* No. 44 strain among the tested strains (Table 2) and the lytic nature was different from those of PLgY, PLgW, and PLgS shown in Table 9. These temperate phages formed very small plaques (about 0.3 mm) than that of virulent phages. The temperate phage induced by UV irradiation also showed the same lysis patterns with those of sea water induced temperate phages.

Fig. 1 shows that the growth of *L. garvieae* No. 16 induced for the temperate phage reached the maximum density of OD_{530nm} 0.6 in sea water-TSB after 7h of incubation and kept at that level for at least 5h. This growth curve was quite different from that of the same strain infected with a virulent phage. It is not clear whether this difference came from the weak infectivity of the temperate phage or remain-

ing of lysogenized cells in the culture. The production of the temperate phage was lower than that (10^{10} PFU/ml) of the virulent phage.

The changes in pathogenicity of *L. garvieae* strains by the induction of the temperate phages should be carefully examined in the future.

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*Lactococcus garvieae*의 새로운 용원성파아지

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TSB 해수배지와 숙주로서 *L. garvieae* No. 44를 배양조건으로 사용한 경우, *L. garvieae* 111균주중 용원화되었다고 추정되어진 96균주의 cells에서 temperate phage가 효과적으로 분리되었다. 하지만 동일한 배양조건에서 보통의 TSB 배지를 사용한 경우에서는 temperate phage는 전혀 나타나지 않았다. 이 temperate phages는 TSB 해수배지와 ultraviolet irradiation를 병용한 경우 역시 효과적으로 분리되었다. 분리된 모든 temperate phages는 기존의 phage(PLgY, PLgW, PLgS) 의 lytic nature와는 달리 오직 *L. garvieae* No. 44만을 lysis하였다. 배양후 약 1시간후 phage가 나타났으며 12시간 후 virulent phage의 최고농도(10^{10} PFU/ml) 보다 훨씬 낮은 농도인 10^{6} PFU/ml까지 증가하였다.

Key words: *Lactococcus garvieae*, Temperate bacteriophage, Lysogeny, Induction of phage, Lytic infection