魚病研究 **Fish Pathology, 52 (2), 82–88, 2017. 6**

Research article

A Novel Plasmid Carrying Capsule Gene Cluster Found in *Lactococcus garvieae* Isolated from Filefish

Kinya Kanai[1]*, Masakazu Tsujikura[2], Kimihiro Shutou[2], Toshio Honma[2], Fumihiro Matsumoto[1], Koushirou Suga[1], Shusaku Takagi[3], Yutaka Fukuda[4] and Yukitaka Sugihara[5]

[1]*Graduate School of Fisheries and Environmental Sciences, Nagasaki University, Nagasaki 852-8521, Japan*
[2]*Bio Science Co. Ltd., Tokushima 779-1292, Japan*
[3]*Fisheries Research Center, Ehime Research Institute of Agriculture, Forestry and Fisheries, Ehime 798-0087, Japan*
[4]*Fisheries Research Division, Oita Prefectural Agriculture, Forestry and Fisheries Research Center, Oita 879-2602, Japan*
[5]*Nagasaki Prefectural Institute of Fisheries, Nagasaki 851-2213, Japan*

(Received December 28, 2016)

ABSTRACT—*Lactococcus garvieae* is recognized as a crucial bacterial pathogen of freshwater and marine fish species. It has been divided into two serological phenotypes, namely KG^- and KG^+. Difference of the two phenotypes is owing to the presence or absence of polysaccharide capsule, and a phenotypic change from KG^- to KG^+ occurs during stocking of isolates for a long period or by repeated subculturing. We found that the phenotypic change occurred more readily in *L. garvieae* isolates from cultured filefish, thread-sail filefish *Stephanolepis cirrhifer* and black scraper *Thamnaconus modestus*, than those from other fish species. Thus we studied the gene cluster for capsular polysaccharide biosynthesis (capsule gene cluster) of a filefish isolate, strain BSLG13015, and revealed that the strain possessed the same capsule gene cluster as those from other fish species, but that it was integrated in a newly identified plasmid. The plasmid, a size of 31,654 bp and circular, was named pBSLG13015. It was detected in all of KG^- filefish isolates but not in KG^+ filefish isolates or *L. garvieae* from other fish species. It is highly probable that the easier change from KG^- to KG^+ in *L. garvieae* filefish isolates is attributed to the loss of the plasmid.

Key words: *Lactococcus garvieae*, capsule gene cluster, plasmid, filefish, KG^-, KG^+, phenotypic change

Lactococcus garvieae is one of the crucial bacterial pathogens of freshwater and marine fish species cultured in the world and is also known to cause diseases in cows and humans (Vendrell *et al*., 2006). In Japan yellowtail *Seriola quinqueradiata* aquaculture industry had frequently suffered great losses due to lactococcosis caused by *L. garvieae* (Kusuda *et al*., 1991), but the losses have been drastically decreased after commercial vaccines became available. Recently, cultivation of filefish, thread-sail filefish *Stephanolepis cirrhifer* and black scraper *Thamnaconus modestus,* has been conducted in Japan, but these species are often suffering from lactococcosis (Minami *et al*., 2012).

In bacterial pathogens such as lactococci and streptococci, cell-surface polysaccharide capsules act as virulence factors but also antigens for serotype differentiation and vaccines. *L. garvieae* is known to produce a capsule (Yoshida *et al*., 1997). Japanese *L. garvieae* isolates derived from diseased fish have been classified into two serotypes, serotype I and II (Fukuda *et al*., 2015; Oinaka *et al*., 2015), and serotype I has been divided into two phenotypes, namely KG^- and KG^+ (Kitao, 1982). Cells of KG^- type are encapsulated and virulent, whereas those of KG^+ type are not encapsulated and nonvirulent (Yoshida *et al*., 1997; Kawanishi *et al*., 2006). These two phenotypes can be differentiated by an agglutination test using KG^- and KG^+ antisera (Kitao, 1982). *L. garvieae* KG^- type possesses a gene cluster for capsular polysaccharide biosynthesis (capsule gene cluster) on the chromosome like other lactococci and streptococci, but KG^+ type does not (Miyauchi *et al*., 2012). This gene cluster is sandwiched between two IS*982* elements. In addition, it is known that a

* Corresponding author
E-mail: kanai@nagasaki-u.ac.jp

Table 1. Origin of *Lactococcus garvieae* strains used in this study

Strain	Phenotype	Fish	Prefecture	Year
NUF1134	KG^-	Thread-sail filefish	Oita	2013
BSLG13015	KG^-	Thread-sail filefish	Oita	2013
BSLG14203	KG^-	Thread-sail filefish	Oita	2014
BSLG14208	KG^-	Thread-sail filefish	Oita	2014
S14-10	KG^-	Thread-sail filefish	Ehime	2014
10/20-1	KG^-	Black scraper	Ehime	2014
120972	KG^+	Thread-sail filefish	Oita	2012
120973	KG^+	Thread-sail filefish	Oita	2012
131611	KG^+	Thread-sail filefish	Oita	2013
131613	KG^+	Thread-sail filefish	Oita	2013
BSLG13013	KG^+	Thread-sail filefish	Oita	2013
S14-20	KG^+	Black scraper	Ehime	2014
BY-1	KG^-	Yellowtail	Nagasaki	1992
KG9502	KG^-	Yellowtail	Kagoshima	1995
NUF752	KG^-	Yellowtail	Nagasaki	1995
NUF1051	KG^-	Yellowtail	Nagasaki	2007
NUF1070	KG^-	Yellowtail	Nagasaki	2008
NUF1019	KG^-	Greater amberjack	Kagoshima	2005
KRS02016	KG^-	Japanese flounder	Kagawa	2002
Ns12sousui54	KG^-	Japanese flounder	Nagasaki	2012
Ns12sousuiVH81	KG^-	Pacific bluefin tuna	Nagasaki	2012
NUF281	KG^+	Yellowtail	Nagasaki	1986
NUF552	KG^+	Yellowtail	Kagoshima	1991
NUF699	KG^+	Japanese flounder	Nagasaki	1993

Thread-sail filefish, *Stephanolepis cirrhifer*; black scraper, *Thamnaconus modestus*; yellowtail, *Seriola quinqueradiata*; greater amberjack, *Seriola dumerili*; Japanese flounder, *Paralichthys olivaceus*; Pacific bluefin tuna, *Thunnus orientalis*.

phenotypic change from KG^- to KG^+ occurs during stocking of isolates for a long period or by repeated subculturing but more rapidly by subculturing on TTC (2, 3, 5-triphenyltetrazolium chloride) -supplemented agar media (Kitao, 1982). So, it was speculated that KG^+ strains might have lost the capsule gene cluster during its subculturing (Miyauchi *et al*., 2012).

L. garvieae isolated from filefish also has been divided into the two serological phenotypes. However, unlike *L. garvieae* isolates from other fish species, when single KG^- colonies of filefish isolates were spread on agar media, KG^+ colonies always grew together with KG^- colonies. Thus the phenotypic change from KG^- to KG^+ in filefish isolates seemed to be more readily occurred than those from other fish species. To elucidate the mechanisms for this phenomenon we conducted a genetic analysis of the capsule gene cluster of filefish isolates in comparing with those of isolates from other fish species.

Materials and Methods

Bacteria

L. garvieae strains used in this study are listed in Table 1. Their serological phenotypes were determined by a slide agglutination test using KG^- and KG^+ rabbit antisera raised against NUF1019 and NUF552, respectively. The strains were cultured on Todd Hewitt (TH) agar (Difco) at 27°C and stocked by freezing at –80°C in a deep freezer or at –196°C in liquid nitrogen.

Change of serological phenotype during cultivation

Single KG^- colonies of KG^- strains derived from filefish, NUF1134, BSLG14208, S14-10 and 10/20-1, and from other fish species, BY-1, KG9502 and Ns12sousui54, were picked from overnight cultures on TH agar plates, suspended in TH broth and spread onto new agar plates. The plates were incubated at 27°C for 3 days, during this period a single KG^- colony of each strain was picked every day from the respective plate and spread onto a new agar plate. After incubating the plates overnight, 10 to 50 colonies grown on each plate were picked and their serological phenotypes were determined by a slide agglutination test.

Detection of capsule gene cluster

L. garvieae capsule gene cluster was amplified using a long PCR method described by Miyauchi *et al*. (2012) with some modifications. Briefly, genomic DNA of each *L. garvieae* strain was purified using Wizard Genomic DNA Purification kit (Promega) according to the manufacturer's instructions. Forward

(5′-CCATTCCGACACGAAACTGATC-3′) and reverse (5′-ATCACTACGGTTGAGGATCATG-3′) primers (Lg-Cps-38F and Lg-Cps-36R, respectively) were newly designed from the *L. garvieae* Lg2 DNA complete genome sequence (GenBank accession no. NC_017490) near those described by Miyauchi *et al*. (2012). PCR was performed on C1000 Thermal Cycler (Bio-Rad) using KOD FX Polymerase (Toyobo). The PCR reaction mixture (50 μL) contained 25 μL of 2× PCR buffer for KOD FX (Toyobo), 0.4 mM of each dNTP, 0.3 μM of each primer, 1 μL of template DNA and 1 U of KOD FX Polymerase. The PCR condition was as follows; initial denaturation at 94°C for 2 min and 30 cycles of denaturation at 98°C for 10 s, annealing at 60°C for 30 s and extension at 68°C for 17 min. The PCR products were electrophoresed in 0.8% agarose gel with Quick Load 1 kb Extend DNA Ladder (New England BioLabs) and stained with ethidium bromide.

Genetic analysis of capsule gene cluster of L. garvieae *BSLG13015*

The capsule synthetic gene locus of *L. garvieae* BSLG13015 derived from thread-fin filefish was fragmentally amplified by PCR and sequenced. Primers used for PCR and sequencing are designed from the database mentioned above. The PCR reaction mixture (50 μL) contained 10 μL of 5× PrimeSTAR Buffer (Takara), 0.2 mM of each dNTP, 0.3 μM of each primer, 1 μL of template DNA and 1.25 U of PrimeSTAR HS Polymerase (Takara). The PCR condition was as follows; initial denaturation at 95°C for 3 min and 30 cycles of denaturation at 98°C for 10 s, annealing at appropriate temperature for 15 s and extension at 72°C for 1 min/kbp. The annealing temperature varied according to the Tm values of primers used. The PCR products were electrophoresed in 0.8% agarose gel, stained with ethidium bromide and extracted from gel using QIAEX II Gel Extraction kit (Qiagen) according to the manufacturer's instructions. Sequencing of extracted DNA was carried out with BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and ABI PRISM 3130 or 3130*xl* Genetic Analyzer (Applied Biosystems).

Upstream and downstream regions of the capsule gene cluster of BSLG13015 were amplified and sequenced using an inverse PCR technique (Ochman *et al*., 1988). Genomic DNA of BSLG13015 was digested with each of restriction enzymes (*Eco*RI, *Hin*dIII, *Pst*I and *Xba*I) and self-ligated using DNA Ligation kit <Mighty Mix> (Takara). Inverse PCR was performed using PrimeSTAR GXL DNA Polymerase (Takara) with the self-ligated (looped) DNA and an outward directed primer pair designed from the sequences near the end of the capsule gene cluster and each restriction site. The PCR reaction mixture (50 μL) contained 10 μL of 5× PrimeSTAR GXL Buffer (Takara), 0.2 mM of each dNTP, 0.3 μM of each primer, 1 μL of template DNA and 1.25 U of PrimeSTAR GXL Polymerase (Takara). The PCR condition was the same as above except that the extension time was fixed for 6 min. The PCR products were extracted from electrophoresis gel and sequenced as above.

DNASIS software (Hitachi software) and BLAST program at National Center for Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast.cgi) were used for predicting open reading frames and homology search, respectively. ClustalW2.1 network version at DNA Data Bank of Japan (http://clustalw.ddbj.nig.ac.jp/index.php?lang) was used for DNA multiple sequence alignment. The sequence data of pBSLG13015 are available on DDBJ/GenBank/EMBL databases under accession number LC205736.

Detection of plasmid pBSLG13015

The presence of pBSLG13015 in the genomes of *L. garvieae* strains from different fish species was investigated by the PCR method using KOD FX Polymerase mentioned above. Primer pairs used for amplification were Lg-pF2 (5′-GTGCCATAAACATCGTCTGGTTG-3′) and Lg-pR2 (5′-ACCAACAGCGACAATAATGACG-3′) for the region containing the capsule gene cluster and Lg-Cps-1F (5′-AGCAGCGTAGGCTATAGAAGCA-3′) and Lg-Cps-37R (5′-TCTTGTCCCAGAGGGTTCTCCT-3′) for the region other than the capsule gene cluster. These two primer pairs were designed so as to amplify the two DNA regions that covered the whole region of the plasmid and, moreover, the both ends of which overlapped each other.

Virulence test

L. garvieae KG9502 (KG^- type) isolated from yellowtail and BSLG13015 and BSLG13013 (KG^- and KG^+ type, respectively) from thread-sail filefish were cultured on TH agar for 24 h. For culturing BSLG13015 a single KG^- colony was picked and inoculated on TH agar. The bacterial suspensions were prepared in 0.01 M phosphate buffered saline, pH7.2 (PBS), serially tenfold-diluted with PBS and injected intramuscularly to ten yellowtail (104.7 ± 19.9 g body weight) and nine thread-sail filefish (50.6 ± 11.8 g body weight) per dilution. After inoculation, experimental fish received the same dilution were stocked in a 100-L circular and 25-L rectangular plastic tank for yellowtail and thread-sail file fish, respectively, with constant water flow and reared with feeding of commercial pelleted diet (3% body weight) every 3 days for 2 weeks. The average water temperature was 24.3°C. Bacterial isolation from the kidney of dead and survived fish was carried out, and serological phenotypes of the isolates were determined by a slide agglutination test described above.

Results

Change of serological phenotype during cultivation

KG⁻ colonies of filefish isolates grown on TH agar plates were larger and more opaque than KG⁺ colonies (Fig. 1). The relationship between cultivation time and the phenotypic change of KG⁻ strains is shown in Fig. 2. When single KG⁻ colonies of strains from filefish were spread onto TH agar plates and cultivated for 1, 2 and 3 days, 4.7–10.8%, 17.9–41.0% and 12.8–35.4% of the colonies grown were revealed to be KG⁺ type, respectively. On the other hand, in case of KG⁻ strains from other fish species none of the colonies grown were changed to KG⁺ type.

Detection and characterization of capsule gene cluster of L. garvieae *BSLG13015*

In a preliminary experiment, *L. garvieae* capsule synthetic genes were detected by PCR, one by one, in the genomic DNA of BSLG13015, suggesting the presence of a capsule gene cluster similar to those of other fish species. However, the capsule gene cluster was not detected in BSLG13015 by the long PCR for amplifying the whole region (Fig.3), indicating that the gene cluster was located at a different place of the genome. Thus we employed an inverse-PCR method to amplify the upstream and downstream regions of the capsule gene cluster to reveal its integrated site. As a result of sequencing the inverse-PCR products, novel sequences, not present in the *L. garvieae* Lg2 genome, were revealed, and their tips were connected, suggesting the existence of a circular structure containing the capsule gene cluster.

The prediction of open reading frames and BLAST homology search demonstrated 32 genes in the circular DNA, including the capsule gene cluster consisted of 15 genes, three IS*982* elements, two of which sandwiched the capsule gene cluster, *repA*, *repB, parA* and genes for transposase, nickase and resolvase but not for drug resistance. The downstream IS*982* element was arranged in the reverse direction compared to the chromosomal capsule gene cluster of *L. garvieae* Lg2. A total length of the circular DNA was 31,654 bp. For this circular DNA structure we propose the name pBSLG13015 (Fig. 4).

Detection of pBSLG13015 among strains

All of six KG⁻ strains from filefish showed positive PCR reactions that presumably indicated the existence of pBSLG13015, whereas all of six KG⁺ strains from

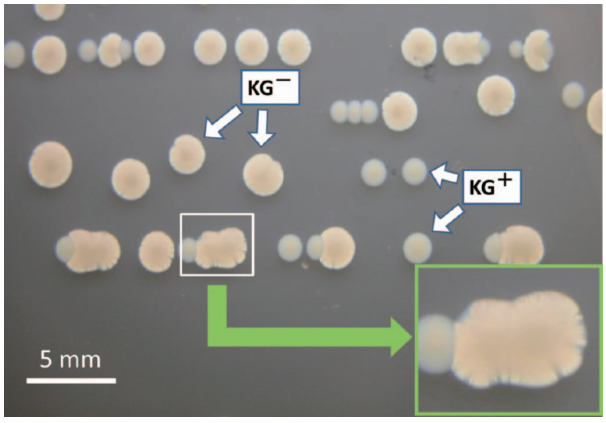

Fig. 1. KG⁻ and KG⁺ colonies of *L. garvieae* BSLG13015 (KG⁻ type) on Todd Hewitt agar cultured for 2 days at 27°C. KG⁻ colonies are relatively large and opaque, whereas KG⁺ colonies are relatively small and transparent. The periphery of KG⁻ colonies seems to be transparent, indicating the transition of colony type from KG⁻ to KG⁺.

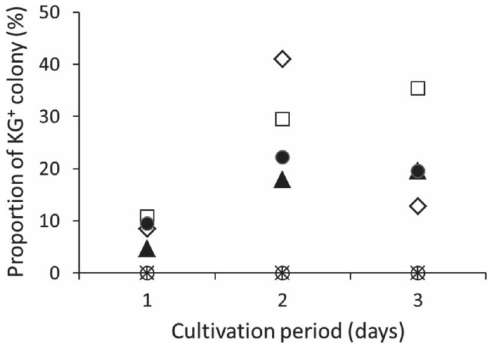

Fig. 2. Proportion of KG⁺ colonies appeared on Todd Hewitt agar inoculated with single KG⁻ 1, 2 and 3 day-cultured colonies of *L. garvieae* KG⁻ strains isolated from filefish (▲, NUF1134; ●, BSLG14208; ◇, S14-10; □, 10/20-1) and other fish species (×, BY-1; ○, KG9502; +, Ns12sousui54).

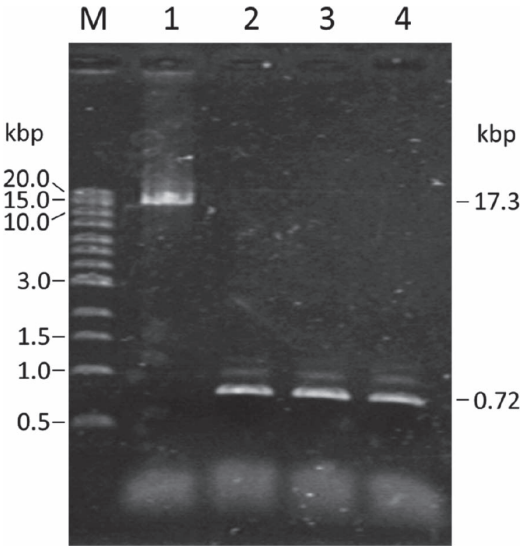

Fig. 3. Detection of the chromosomal *L. garvieae* capsule gene cluster by PCR. Appearance of bands at 17.3 kbp and 720 bp indicates the presence and absence of the gene cluster, respectively. M, DNA size marker; 1, BY-1 (KG⁻ type); 2, NUF552 (KG⁺ type); 3, BSLG13015 (KG⁻ type); 4, BSLG13013 (KG⁺ type).

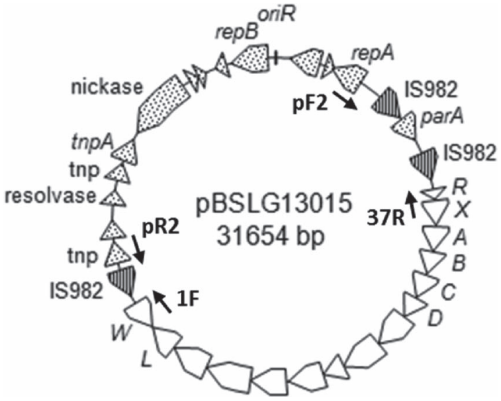

Fig. 4. Structure of the plasmid pBSLG13015. Boxes with hatched line, no pattern and dotted pattern are IS*982* elements, the genes of capsule gene cluster and the other genes, respectively. *R* to *W* represent *epsR*, *epsX*, *epsA*, *epsB*, *epsC*, *epsD*, *epsL* and *epsW* in the chromosomal capsule gene cluster of *L. garvieae* Lg2 described in Miyauchi *et al*. (2012). tnp, transposase. Arrows indicate the position and direction of PCR primers used for detection of the plasmid among the strains: pF2, Cps-pF2; pR2, Cps-pR2; 1F, Lg-Cps-1F; 37R, Lg-Cps-37R.

filefish and 12 strains, both KG^- and KG^+, from other fish species showed negative reactions for the plasmid but positive reactions for the chromosomal capsule gene cluster (Fig. 5). The plasmid was not detected in KG^+ colonies derived from KG^- strains isolated from filefish (Fig. 6).

Virulence of filefish strains against filefish and yellowtail

L. garvieae BSLG13015 (KG^- type) was shown to be virulent against both thread-fin filefish and yellowtail and seemed to be more virulent than *L. garvieae* KG9502 (KG^- type) judged from the time to death. On the other hand, the virulence of *L. garvieae* BSLG13013 (KG^+ type) was low against either fish species (Fig. 7). The serological phenotype of *L. garvieae* isolated from dead fish was the same as that of the inoculated strain. From some survived fish, *L. garvieae* was re-isolated. All the isolates from survived fish inoculated with KG9502 and BSLG13013 were KG^- and KG^+, respectively.

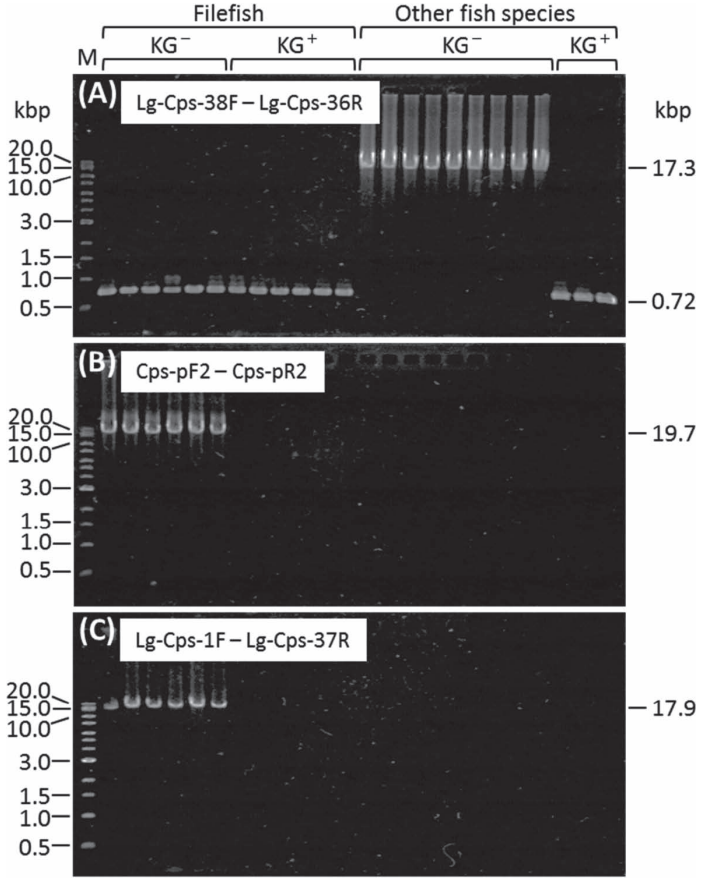

Fig. 5. Detection of the chromosomal capsule gene cluster and pBSLG13015 in the *L. garvieae* strains by PCR. The PCR products of the strains are applied from left to right in the same order as those listed in Table 1. (A) The chromosomal capsule gene cluster. Appearance of bands at 17.3 kbp and 720 bp indicates the presence and absence of the gene cluster, respectively. (B) pBSLG13015 (region containing the capsule gene cluster, 19.7 kbp). (C) pBSLG13015 (region containing genes other than the capsule gene cluster, 17.9 kbp). M, DNA size marker.

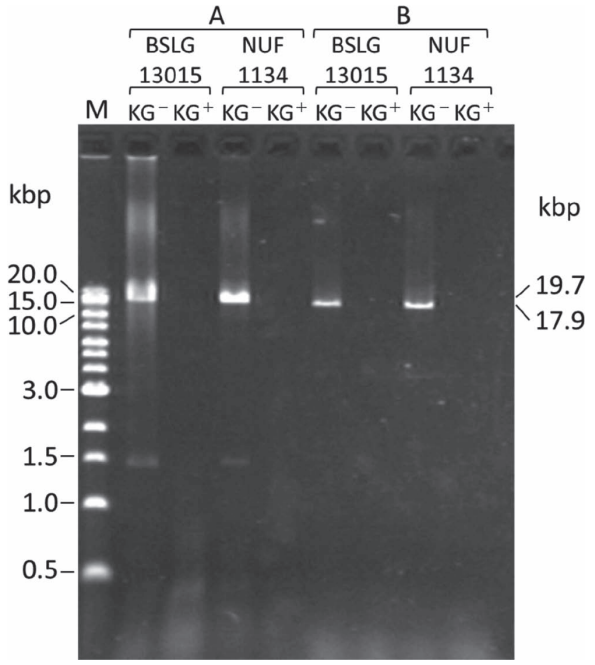

Fig. 6. Detection of pBSLG13015 in the KG^- and KG^+ colonies of *L. garvieae* strains BSLG13015 and NUF1134 by PCR. (A) The region containing the capsule gene cluster (19.7 kbp). (B) The region containing genes other than the capsule gene cluster (17.9 kbp). M, DNA size marker.

Discussion

In this study we demonstrated the presence of a plasmid that carried the capsule gene cluster in *L. garvieae* strains derived from diseased filefish. In pathogenic lactococcal or streptococcal species, to the best of our knowledge, it is the first case that a plasmid carries a gene cluster for polysaccharide capsule synthesis. Sequencing the upstream and downstream regions of the capsule gene cluster of *L. garvieae* BSLG13015 revealed novel nucleotide sequences which did not exist in the *L. garvieae* Lg2 genomic DNA, and their tips were connected to form a circle. Moreover, the newly found sequences contained genes for plasmid replication and several transposases, and approximately 63% of the nucleotide sequence of this region showed a high similarity to *L. lactis* plasmid pK214 (Perreten *et al*., 1997). Thus we concluded that this circular structure is a plasmid. In the genus *Lactococcus* it is known that some species possess a plasmid carrying a gene cluster for exopolysaccharide production, but such bacteria work as a starter culture of fermented dairy products (van Kranenburg *et al*., 2000; Forde and Fitzgerald, 2003; Knoshaug *et al*., 2007). The presence of plasmids carrying a gene cluster for exopolysaccharide production might be a common feature in *Lactococcus*.

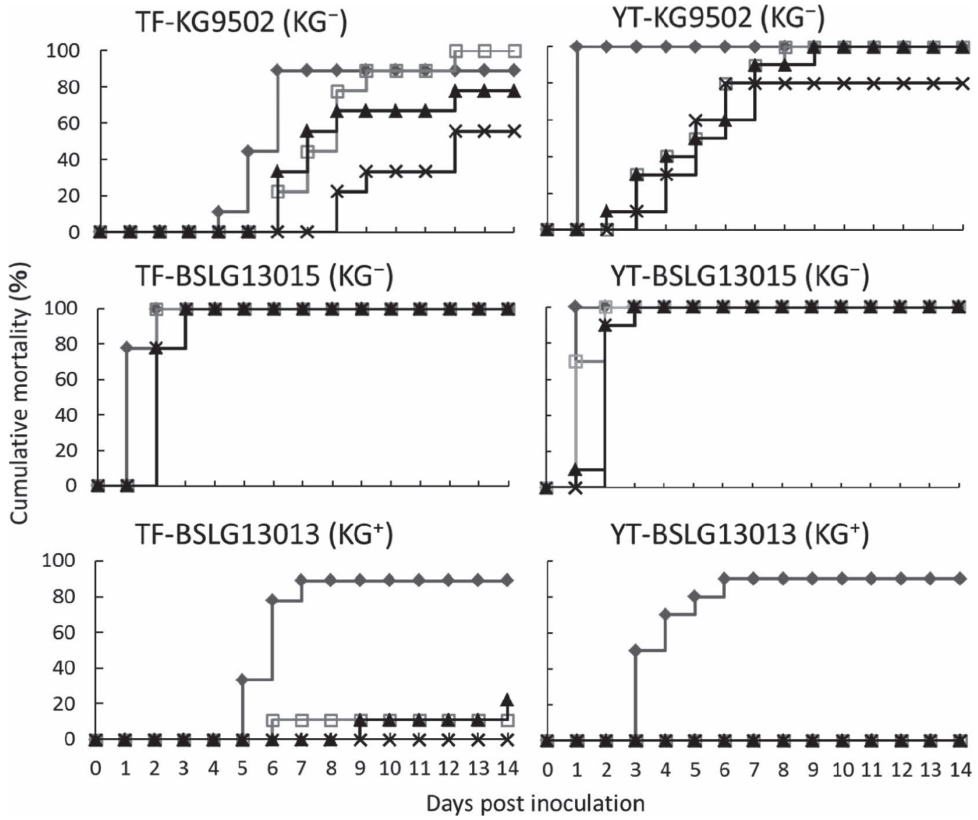

Fig. 7. Virulence of *L. garvieae* strains to thread-sail filefish (TF) and yellowtail (YT) studied by intramuscular inoculation. ◆, 10^8; □, 10^6; ▲, 10^4; ×, 10^2 CFU/100 g body weight.

In this study the plasmid pBSLG13015 was detected in all the KG^- *L. garvieae* strains from filefish tested, but was not detected in those from other fish species. So, it was supposed that *L. garvieae* possessing the plasmid was restricted to filefish, and we speculated that the filefish isolates were pathogenic only to filefish. However, the results of a virulence test conducted in this study showed that the representative strain from filefish seemed to be highly pathogenic both to filefish and yellowtail. We employed intramuscular inoculation method to assess the virulence of the strains. Infection experiments using oral or immersion challenge method are needed for clarifying if the infectivity or entry to fish differs between the isolates from filefish and yellowtail. In addition, it is necessary to ascertain if the presence of the plasmid is restricted to filefish isolates through increasing the number of the isolates examined.

It is highly probable that the change from KG^- to KG^+ phenotype in *L. garvieae* isolates from filefish is due to the loss of the plasmid, because the plasmid was not detected in KG^+ colonies derived from KG^- strains from filefish (Fig. 6). On the other hand, in the case of isolates from other fish species two IS*982* elements located at both sides of the capsule gene cluster may be involved in the deletion of the gene cluster. The phenotypic change from KG^- to KG^+ occurred much more easily in the strains derived from filefish than those from other fish species upon culturing on agar media. Thus curing of the plasmid seems to be more easily occurred than loss of the gene cluster from the chromosome.

When *L. garvieae* isolates from filefish are cultured for a long period of time or subcultured repeatedly on agar media, KG^- colonies may disappear soon. To prevent this, it is recommended that opaque colonies (KG^- colonies) are picked up upon subculturing or that the bacteria are stocked by freezing.

References

Forde, A. and G. F. Fitzgerald (2003): Molecular organization of exopolysaccharide (EPS) encoding genes on the lactococcal bacteriophage adsorption blocking plasmid, pCI658. *Plasmid*, **49**, 130–142.

Fukuda Y, Y. Tue, D. Oinaka, Y. Wada, A, Yamashita, S. Urasaki, S. Yoshioka, K. Kimoto and T. Yoshida (2015): Pathogenicity and immunogenicity of non-agglutinating *Lactococcus garvieae* with anti-KG^- phenotype rabbit serum in *Seriola* spp. *Fish Pathol*., **50**, 200–206.

Kawanishi, M., T. Yoshida, S. Yagashiro, M. Kijima, K. Yagyu, T. Nakai, M. Murakami, H. Morita and S. Suzuki (2006): Differences between *Lactococcus garvieae* isolated from the genus *Seriola* in Japan and those isolated from other animals (trout, terrestrial animals from Europe) with regard to pathogenicity, pharge susceptibility and genetic characterization. *J. Appl. Microbiol*., **101**, 496–504.

Kitao, T. (1982): The methods for detection of *Streptococcus* sp. causative bacteria of streptococcal disease of cultured yellowtail (*Seriola quinqueradiata*). *Fish Pathol*. **17**, 17–26. (In Japanese)

Knoshaug, E. P., J. A. Ahlgren and J. E. Trempy (2007): Exopolysaccharide expression in Lactococcus lactis subsp. cremoris Ropy352: Evidence for novel gene organization. *Appl. Environ. Microbiol*., **73**, 897–905.

Kusuda, R., K. Kawai, F. Salati, C. R. Banner and J. L. Fryer (1991): *Enterococcus seriolicida* sp. nov., a fish pathogen. *Int. J. Syst. Bacteriol*., **41**, 406–409.

Minami, T., M. Kanemaru, K. Iwata, K. Nakanishi, A. Yamashita, Y. Miyoshi, Y. Fukuda and T. Yoshida (2012): Occurrences of diseases in thread-sail filefish in western part of Japan. *Fish Pathol*., **47**, 111–113.

Miyauchi, E., H. Toh, A. Nakano, S. Tanabe and H. Morita (2012): Comparative genomic analysis of *Lactococcus garvieae* strains isolated from different sources reveals candidate virulence genes. *Int. J. Microbiol*., **2012**, article ID 728276, doi: 10.1155/2012/728276.

Ochman, H., A. S. Gerber and D. L. Hartl (1988): Genetic applications of an inverse polymerase chain reaction. *Genetics*, **120**, 621–623.

Oinaka, D., N. Yoshimura, Y. Fukuda, A. Yamashita, S. Urasaki, Y. Wada and T. Yoshida (2015): Isolation of *Lactococcus garvieae* showing no agglutination with anti-KG^- phenotype rabbit antiserum. *Fish Pathol*., **50**, 37–43.

Perreten, V., F. Schwarz, L. Cresta, M. Boeglin, G. Dasen and M. Teuber (1997): Antibiotic resistance spread in food. *Nature*, **389**, 801–802.

van Kranenburg, R., M. Kleerebezem and W. M. de Vos (2000): Nucleotide sequence analysis of the lactococcal EPS plasmid pNZ4000. *Plasmid*, **43**, 130–136.

Vendrell, D., J. L. Balcázar, I. Ruiz-Zarzuela, I. de Blas, O. Gironés and J. L. Múzquiz (2006): *Lactococcus garvieae* in fish: A review. *Comp. Immun. Microbiol. Infect. Dis*., **29**, 177–198.

Yoshida, T., M. Endo, M. Sakai and V. Inglis (1997): A cell capsule with possible involvement in resistance to opsonophagocytosis in *Enterococcus seriolicida* isolated from yellowtail *Seriola quinqueradiata*. *Dis. Aquat. Organ*., **29**, 233–235.