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

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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 IS982 elements. In addition, it is known that a
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 Nuf12sousui54, 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
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 5x 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 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.

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’-GTGCCATAAAACATCGTCTGTTG-3’) and Lg-pR2 (5’-ACCAACAGCGACAATATGACG-3’) for the region containing the capsule gene cluster and Lg-Cps-1F (5’-AGGACGGTGGCTAGATAGCGA-3’) and Lg-Cps-3R (5’-TCTTTGTCACCAGGGTTTCCTC-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.

(5’-CCATTCCGACAGAAGACTGATC-3’) and reverse (5’-ATCATACCGTGGTGAATCATAG-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 Miyazaki 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 2x 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.

DNA Sequence and Multiple Sequence Alignment

The multiple sequence alignment program at National Center for Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was 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.

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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 IS982 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 IS982 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

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**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).
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**

*Ochrobactrum anthropi* 10251 (KG⁻ type) was shown to be virulent against both thread-fin filefish and yellowtail and seemed to be more virulent than *Ochrobactrum anthropi* KG9502 (KG⁻ type) judged from the time to death. On the other hand, the virulence of *Ochrobactrum anthropi* KG9502 (KG⁺ type) was low against either fish species (Fig. 7). The serological phenotype of *Ochrobactrum anthropi* isolated from dead fish was the same as that of the inoculated strain. From some survived fish, *Ochrobactrum anthropi* was re-isolated. All the isolates from survived fish inoculated with KG9502 and BSLG13013 were KG⁻ and KG⁺, respectively.

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**Fig. 4.** Structure of the plasmid pBSLG13015. Boxes with hatched line, no pattern and dotted pattern are IS982 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 *Ochrobactrum anthropi* 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.

**Fig. 5.** Detection of the chromosomal capsule gene cluster and pBSLG13015 in the *Ochrobactrum anthropi* 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.
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. 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.

Fig. 7. Virulence of *L. garvieae* strains to thread-sail filefish (TF) and yellowtail (YT) studied by intramuscular inoculation. ◆, 10⁶; □, 10⁵; ▲, 10⁴; ×, 10² 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 IS982 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**


