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Expression of the serum opacity factor gene and the variation in its upstream region in *Streptococcus dysgalactiae* isolates from fish

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Group C *Streptococcus dysgalactiae* (GCSD) is a pathogen of farmed fish. Almost all GCSD isolates from Asian countries, including Japan, Taiwan, Malaysia, and China, have a serum opacity factor (SOF-FD). Although the SOF-FD sequences in different GCSD isolates are identical, different opacification activities are observed. Three types of variations were observed in the upstream sequence of the *sof-FD* gene in GCSD isolates with different SOF-FD activities. Type 1 was characterized by insertion of an IS981-like element into the upstream region of the *sof-FD* gene. In Type 2, an IS981-like element was inserted into the upstream region in a direction opposite to that in Type 1. In Type 3, no IS element was inserted. Type 1 was predominant among Japanese isolates (129 of 133). Isolates from other Asian countries were generally Type 3 (13 of 16). Except for 1 strain, Type 1 strains exhibited opacification activities with optical densities (ODs) >0.6, while Type 2 and Type 3 strains have low opacification activities (ODs >0.2). Only Type 1 strains have putative –10 and –35 promoter regions upstream of the *sof-FD* gene, and the expression level of the *sof-FD* gene was higher in Type 1 strains than in Type 2 and Type 3 strains.

Key Words—IS981 element; qPCR; SOF; *Streptococcus dysgalactiae*

Introduction

Lancefield group C *Streptococcus dysgalactiae* subsp. *dysgalactiae* (GCSD) has been isolated from farmed amberjack (*Seriola dumerili*) and yellowtail (*Seriola quinqueradiata*) in Japan since 2002 (Nomoto et al.,

2004, 2006). GCSD infections typically cause severe necrosis of the caudal peduncle (Hagiwara et al., 2009, 2010). GCSD is mainly known as a causative agent of mastitis in cattle, endocarditis in domestic animals, and cardiopulmonary diseases or adenoiditis in humans (Aarestrup and Jensen, 1996; Efstratiou et al., 1994). GCSD isolated from fish in Japan is similar, but genetically different from that isolated from mammals (Nishiki et al., 2010). To differentiate fish isolates from mammalian isolates, primer sets targeting the *sodA* and *sof-FD* genes were established (Nishiki et al., 2011; Nomoto et al., 2008).

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GCSD infections in farmed fish have also been reported in other Asian countries, including China, Indonesia, Malaysia, and Taiwan (Abdelsalam et al., 2010a; Yang and Li, 2009).

In a previous study, serum opacity factor (SOF) was detected in GCSD isolated from fish (Nishiki et al., 2011). The SOF obtained from fish isolates, named SOF-FD, consists of a putative signal sequence, a large N-terminal opacification domain, a putative fibronectin-binding domain, and an LPXTG Gram-positive anchor motif. The structure of SOF-FD is similar to that of the SOF in *S. pyogenes* (Nishiki et al., 2011). The substrate of SOF is a high density lipoprotein (HDL) which has anti inflammation activity (Rye and Barter, 2008). The SOF binds to HDL and then disrupts its structure, which may contribute to the virulence of streptococci (Courtney et al., 2006). In the previous studies of *S. pyogenes* and *S. suis*, SOF is an important virulence determinant and a candidate for vaccine antigen (Baums et al., 2006; Courtney et al., 1999; Gillen et al., 2008; Timmer et al., 2006). One-half of *S. pyogenes* clinical isolates have a SOF, and the sequence of the N-terminal opacification domain is variable (Beall et al., 2000; Goodfellow et al., 2000). In contrast, nearly all GCSD strains isolated from fish have serum opacification activity and *sof-FD* genes with highly homogeneous sequences (Nishiki et al., 2011). In fish isolates, various opacification activities were observed, although their SOF-FD amino acid sequences were identical (Nishiki et al., 2011). Therefore, to elucidate the mechanism underlying the various

opacification activities observed in fish GCSD, we analyzed the sequence of the upstream regions of the *sof-FD* gene, including the putative promoter sequence, as well as *sof-FD* gene expression.

Materials and Methods

Bacterial strains and growth conditions. A total of 133 fish isolates from Japan and 16 fish isolates from other Asian countries were used in this study (Table 1). Lancefield streptococcal grouping (Lancefield, 1933) was performed for these isolates using the Pastorex Strep test (Bio-Rad, Marnes-la-Coquette, France) according to the manufacturer's protocol. All isolates were identified as GCSD using a PCR assay targeting the 16S-23S rDNA spacer region (Forsman et al., 1997; Hassan et al., 2003) and the *sodA* gene (Nomoto et al., 2008). In addition, the *sof-FD* gene was detected in all the isolates using *sof-FD* specific primers as described previously (Nishiki et al., 2011). The isolates were cultured in Todd-Hewitt (TH) broth or agar (Difco, Sparks, MD, USA) at 37°C.

Serum opacification activity. Serum opacification activity was examined using the microtiter plate method as described previously (Nishiki et al., 2011). Briefly, bacterial strains were cultured in TH broth at 37°C for 24 h. One milliliter of the cultured broth was centrifuged, and the bacterial cell pellet was resuspended in 300 µl of physiological saline (0.85% NaCl) containing sodium dodecyl sulfate (SDS) at a final concentration of 0.5%. The suspension was incubated at 37°C for 1 h

Table 1. GCSD isolates used in this study and the results of the *sof-FD* typing and opacification activity assays.

Strain no.	Country of origin	Year of isolation	Source	<i>sof-FD</i> typing	
08-29	Japan	2002	Amberjack	1	OD > 0.6
Kdys0603	Japan	2006	Amberjack	1	
Other Japanese isolates (n=127)	Japan	2002-2010	Amberjack	1	
AOD96086-K	Taiwan	2007	Cobia	1	
WSSN1609	Malaysia	2004	Snapper	1	
PP1635	China	2005	Pompano	1	
04082601F	Japan	2004	Amberjack	2	OD < 0.2
TM03901	Japan	2003	Amberjack	2	
KC041005	Japan	2004	Amberjack	2	
KC03917	Japan	2003	Amberjack	3	
94414	Taiwan	2005	Mullet	3	
Other Taiwanese isolates (n=9)	Taiwan	2005-2007	Mullet	3	
PP880	Malaysia	2003	Pompano	3	
PP1398	Malaysia	2005	Pompano	3	
PP1564	China	2005	Pompano	3	
Kdys0531	Japan	2005	Amberjack	1 (variant)	No activity

and then passed through a 0.45- μ m filter (Sartorius Stedim Japan K. K., Japan). Ten microliters of the SDS extract was mixed with 100 μ l of pooled fish serum from seven healthy amberjack individuals (average body weight, 1.5 kg). Physiological saline containing 0.5% SDS was also mixed with fish serum as a control. Subsequently, the mixture was incubated at 37°C for 24 h. Serum opacification activity was determined on the basis of the optical density (OD) at 405 nm measured using a microplate reader. When the OD of a sample was larger than that of the control by 0.6 or greater, the opacification activity of the sample was considered strong. In contrast, when the difference in the OD of a sample and the control was less than 0.2, the opacification activity of the sample was considered weak. All serum opacification activity experiments were performed in triplicate.

Determination of the *sof-FD* gene sequence and its upstream regions. Table 2 and Fig. 1 show the oligonucleotide primers used in this study and their positions, respectively. To determine the upstream regions of the *sof-FD* gene, 08-29 and Kdys0603, with opacification activities OD > 0.6; TM03901, 04082601F, KC03917, and 94414, with opacification activities OD < 0.2; and Kdys0531, which had no opacification activity; were selected as representative strains. The *sof-FD* gene and its upstream regions were amplified using the GenomeWalker™ Universal Kit (Clontech Laboratories, Inc., Palo Alto, CA, USA) according to the manufacturer's protocol. DNA manipulation, cloning, and sequence analysis were performed as described previously (Nomoto et al., 2008).

PCR assay for *sof-FD* genotyping. Three PCR primer sets were designed to identify three unique upstream sequences (Table 2). SOF-type1 and SOF-54 were used for Type 1, SOF-type2 and SOF-54 were used for Type 2, and SOF-type3 and SOF54 were used for Type 3. All the isolates were subjected to PCR us-

Fig. 1. Schematic representation of the three types of the *sof-FD* genes and upstream regions.

The black arrow indicates the position of a primer. The underlined nucleotide sequence represents the IS981 inverted repeat. The putative -35 and -10 regions are shown in bold.

ing the above primer sets to amplify Types 1, 2, and 3. The amplification program for all three primer sets consisted of an initial denaturation at 95°C for 3 min, then 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1.5 min, and a final extension at 72°C for 5 min.

Quantitative reverse transcription (RT)-PCR (qPCR). For quantitative RT-PCR (qPCR), Type 1 (08-29 and Kdys0603), Type 2 (TM03901 and 04082601F) and Type 3 (KC03917 and 94414) strains were used as representative strains. These strains were cultured in TH broth at 37°C until they reached mid-exponential phase (OD₆₀₀=0.3–0.4). One milliliter of each culture was inoculated into 30 ml of fresh TH broth, and the fresh TH

Table 2. Oligonucleotide primers used in this study.

Target gene	Product size (bp)	Forward primer	Reverse primer
<i>sof-FD</i> Type 1	1,226	SOF-type1;TCATTCTGGTCAATCCGTCA	
<i>sof-FD</i> Type 2	824	SOF-type2;CCCAAATCCCAAGCAATG	SOF-54*;AACGGAGACAAGACCGACAG
<i>sof-FD</i> Type 3	392	SOF-type3;GGGAGAGTGTGCTGACAAATG	
For qPCR assay			
16S rRNA	160	16Sq1;CTCGTGTGCTGAGATGTTGG	16Sq2;TGTGTAGCCCAGGTCATAAGG
<i>sof-FD</i>	160	SOFq1;AGGCTGGATGGCTTGATG	SOFq2;TGGGCTGAAAGATTATGTTG

*SOF-54 was used as the reverse primer for SOF-type1, SOF-type2, and SOF-type3.

broth was incubated at 37°C for 6 h. After incubation, 3 ml of the incubated broth was divided into two 1.5-ml tubes, and the bacterial cells in the two tubes were collected as two cell pellets by centrifugation. One cell pellet was re-suspended in 300 µl of 0.5% SDS in physiological saline to examine the opacification activity as described above. The other cell pellet was used for RNA extraction and cDNA synthesis.

RNA was extracted using a High Pure RNA Isolation Kit (Roche Molecular Biochemicals, Switzerland) following the manufacturer's protocol. Subsequently, cDNA was synthesized using a ReverTra Ace® qPCR RT Kit (Toyobo, Japan) according to the manufacturer's protocol. The qPCR assay was performed using the Thunderbird® SYBR® qPCR Mix (Toyobo, Japan). The 16S rRNA gene was used as an internal control to assess gene expression. The primer sets targeting the 16S rRNA and *sof-FD* genes for qPCR are listed in Table 2. The reactions were performed in the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, USA), and the conditions for qPCR were 95°C for 1 min followed by 40 cycles of 95°C for 15 s, 56°C for 20 s, and 60°C for 40 s. Relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001). The serum opacification activity and qPCR assays were performed in triplicate. The serum opacification activity and the gene expression level of the representative strains were analyzed using Student's *t* test.

Nucleotide sequence accession numbers. The GenBank accession numbers for the entire open reading frame sequences of the *sof-FD* gene and its upstream sequences in 08-29, Kdys0603, TM03901, 04082601F, KC03917, 94414, and Kdys0531 are AB714581, AB695259–AB695262, AB714580, AB695264, respectively.

Results and Discussion

Serum opacification activity

SDS extracts obtained from 129 isolates from Japan and three isolates from other Asian countries exhibited strong serum opacification activities, with OD values above 0.6. Conversely, only four isolates from Japan and 13 isolates from other Asian countries exhibited weak opacification activities (OD < 0.2). No opacification activity was observed in the Kdys0531 isolate from an amberjack in Japan (Table 1).

Sequence determination of the *sof-FD* gene

High similarity (>99%) was observed throughout the entire *sof-FD* gene sequence among the 08-29, Kdys0603, 04082601F, TM03901, KC03917, and 94414 strains. The amino acid sequences of the SOF-FD opacification domains were completely identical.

The sequence of the *sof-FD* gene in the Kdys0531 strain, which had no opacification activity, was determined. Analysis showed that a putative IS1161-like element was inserted into the *sof-FD* gene in the SOF-FD opacification domain (shown as a Type 1 variant in Fig. 1). Therefore, Kdys0531 could not synthesize mature SOF-FD, which resulted in a loss of opacification activity.

Determination of the sequence of the upstream region of the *sof-FD* gene

To elucidate the mechanism underlying various opacification activities, the upstream region of the *sof-FD* gene was examined. Figure 1 shows a schematic representation of the upstream sequences of the *sof-FD* gene. Three different types of *sof-FD* upstream regions were observed in this study. Type 1, which was seen in the 08-29, Kdys0603, and Kdys0531 strains, was characterized by the insertion of a *Lactococcus lactis* IS981 family sequence into the 53-bp region upstream of the *sof-FD* start codon. Putative –10 (TAAAT) and –35 (TTGACT) regions for a promoter sequence were observed upstream of the *sof-FD* gene. The putative –35 region was located within imperfect inverted repeats of the IS981-like element, while the putative –10 region was observed outside the IS981-like element. In Type 2, which was observed in the TM03901 and 04082601F strains, an IS981-like element was also inserted into the upstream region of the *sof-FD* gene; however, its direction was opposite to that in Type 1. Therefore, a putative –35 region corresponding to the –10 region could not be detected. In contrast, Type 3, which was seen in the KC03917 and 94414 strains, had no insertion sequence in the upstream region of the *sof-FD* gene. The sequence of the hypothetical phage protein in the upstream region of the IS981-like element in Type 1 was identical to that of the *sof-FD* gene in Type 3.

qPCR and SOF activity analysis

Expression of the *sof-FD* gene and SOF activity were investigated in six representative strains belonging to three *sof-FD* types. Figure 2 shows the expression of

Fig. 2. The expression of *sof-FD* relative to 16S rRNA and the opacification activity of Type 1, 2, and 3 strains.

For each strain, total RNA was prepared for the qPCR assay together with the SDS extract used for the opacification activity. *The expression level of the *sof-FD* gene and the opacification activity were significantly higher in the Type 1 strains than in Types 2 and 3 strains ($p < 0.01$).

sof-FD relative to that of 16S rRNA and the opacification activity of the six strains. Expression of the *sof-FD* gene and serum opacification activity were significantly higher in the two Type 1 strains (08-29 and Kdys0603) than in the two Type 2 strains (04082601F and TM03901) and the two Type 3 strains (KC03917 and 94414) ($p < 0.01$). Therefore, the differences in the upstream regions may lead to dissimilarities in *sof-FD* gene expression and SOF activities.

In general, the polar effect mediated by IS insertion is involved in gene expression (Jordan et al., 1968). In a previous study, IS981-mediated transcription of the *ldhB* gene was reported in a lactate dehydrogenase-deficient strain of *Lactococcus lactis* (Bongers et al., 2003). In this case, the -35 region (TTGACT) derived from the IS981-like element was believed to act with the -10 region (TAAAT) derived from the native sequence of the *ldhB* gene, resulting in modulation of gene expression. Using a similar mechanism, only Type 1 strains could adequately express the *sof-FD* gene. One Japanese isolate, Kdys0531, was identified as SOF negative, and this isolate possessed an *sof-FD* gene in which the IS1161-like element was inserted near the center of the opacification domain. Due to this insertion, Kdys0531 could not synthesize mature SOF-FD. IS981, IS1161, or IS981-IS1161 hybrid sequences were observed at the end of the *spegg* gene open reading frame in fish GCSD isolates (Abdelsalam et al., 2010b). Hence, some copies of IS981 or IS1161-

like elements likely exist and these factors might affect the expression of other genes in fish GCSD isolates.

PCR assay for *sof-FD* genotyping

Three oligonucleotide primer sets were used in the PCR assay to discriminate between Types 1, 2, and 3. Figure 3 shows the results obtained from this PCR assay. In Type 1, when SOF-type1 and *sof-FD* primers were used, approximately 1,200-bp PCR products were observed in almost all of the Japanese isolates (129/133) and in three isolates from other Asian countries. Only three Japanese isolates were identified as Type 2, and when SOF-type2 and SOF-53 primers were used, PCR products of nearly 800 bp were observed. When the SOF-type3 and SOF-53 primers were used, one Japanese isolate and 13 isolates from other Asian countries were identified as Type 3; as evidenced by the amplification of approximately 400 bp fragments. All the Type 1 strains produced PCR products of nearly 1,600 bp by SOF-type 3 and SOF-54 primer pair, because these isolates had identical sequence to Type 3 strains upstream of IS981.

Table 1 shows the results of *sof-FD* typing and the opacification activities of the GCSD isolates used in this study. As shown in this table, the OD values of the isolates belonging to Type 1 were higher than 0.6, while those of isolates belonging to Type 2 and Type 3 were lower than 0.2. Type 1 was dominant in Japanese isolates, while Type 3 was mainly observed in isolates from other Asian countries, particularly Taiwan. The upstream sequence of the IS981-like element in Type 1 strains and that of the *sof-FD* gene in Type 3 strains were identical. These results indicated that GCSD isolates might be prone to insertion or removal of the

Fig. 3. Typing of *sof-FD* upstream region by PCR assays.

Genomic DNA extracted from all GCSD isolates was examined with three different primer sets (Lane 1, SOF-type 1 and SOF-54 for the Type 1 strains; Lane 2, SOF-type 2 and SOF-54 for the Type 2 strains; and Lane 3, SOF-type 3 and SOF-54 for the Type 3 strains).

IS981-like element. Insertion of the IS981-like element was observed in *L. lactis* when the strain was subcultured under anaerobic conditions (Bongers et al., 2003). Therefore, subculture of Type 3 strains in fish bodies or fish serum could cause insertion of the IS981-like element into the upstream region of the *sof-FD* gene. However, SOF activity could not be recovered when Type 2 and Type 3 isolates were successively subcultured for 30 generations on TH agar containing 10% fish serum (data not shown).

SOF was reported as an important virulence determinant of *S. pyogenes* and *S. suis* (Baums et al., 2006; Courtney et al., 1999). In *S. suis*, IS-like elements might contribute to the allelic variation in the *ofs* gene and disturb the synthesis of intact OFS protein (Takamatsu et al., 2008). The IS-like element was often reported as an important factor allowing microbes to survive in various environments (Duwat et al., 1997; Papadopoulos et al., 1999; Riehle et al., 2001). Because normal expression of the *sof-FD* gene in Japanese isolates was mediated by IS981-like elements, SOF-FD might play an important role as a virulence determinant in the survival of GCSD against fish immune response. Further studies are required to investigate SOF-FD as a virulence determinant in GCSD isolated from farmed fish.

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