Research article

Existence of Subserotypes in *Streptococcus parauberis* Serotype I

Kinya Kanai[*], Chuandeng Tu, Naoki Katayama and Koushirou Suga

*Graduate School of Fisheries Science and Environmental Studies, Nagasaki University,
Nagasaki 852-8521, Japan*

ABSTRACT—*Streptococcus parauberis* is the etiologic agent of streptococcosis in Japanese flounder *Paralichthys olivaceus*.   Two serotypes, termed serotypes I and II, are known among the Japanese *S. parauberis* isolates.   In the course of serodiagnosis, we found several strains that did not agglutinate with rabbit anti-serotype I or II sera.   In this study, we investigated the serological and genetic relationships among the stocked *S. parauberis* strains including the non-agglutinating ones using a newly prepared rabbit antiserum against a non-agglutinating strain (NUF1071) as well as previously prepared antiserotype I and II sera, and pulsed-field gel electrophoresis (PFGE).   An antiserum cross-absorption test and microtiter agglutination test revealed that the serotype I was divided into three subserotypes, tentatively designated Ia, Ib and Ic.   The non-agglutinating strains belonged to the subserotype Ic.   Of the 104 serotype I strains, 6, 91 and 7 strains belonged to subserotypes Ia, Ib and Ic, respectively.   Formalin-killed cells of subserotype Ia and Ic strains were agglutinated with the anti-subserotype Ia serum (so far being used as an anti-serotype I serum) and Ic serum, respectively.   Subserotype Ib strains were agglutinated with both sera.   In PFGE analysis, the stocked 188 *S. parauberis* strains were divided into three clusters corresponding to subserotypes Ib/Ic, Ia and serotype II.

Key words: *Streptococcus parauberis*, serotype, subserotype, PFGE, *Paralichthys olivaceus*, Japanese flounder

Streptococcus parauberis is the etiologic agent of streptococcosis in fish as well as mastitis in cows (Williams and Collins, 1990; Doménech *et al*., 1996). In Japan this bacterium has been recognized as a cause of the disease in Japanese flounder *Paralichthys olivaceus* since 2002 (Kanai *et al*., 2009).   The slide agglutination test using rabbit antisera divided strains that were isolated from flounder into two serotypes, which were termed serotypes I and II (Kanai *et al*., 2009).   Interestingly, all the serotype II strains were found to be tetracycline-resistant due to harboring a *tet*(M) gene on a Tn*916*-like element, and several serotype I strains were also tetracycline-resistant due to harboring a *tet*(S) gene on an 11-kbp plasmid (Meng *et al*., 2009a, b).   Accordingly, the drug susceptibility of *S. parauberis* isolates can be estimated to some extent by conducting serodiagnosis using anti-serotype I and II sera.

In the course of serodiagnosis we found *S. parauberis* isolates that did not agglutinate with either of the anti-serotype I and II sera in 2008.   The non-agglutinating isolates were identified as *S. parauberis* by

species-specific PCR (Mata *et al*., 2004).   Since then non-agglutinating strains were occasionally found.   In this study, we reexamined the serological relationship among the stocked *S. parauberis* strains including the non-agglutinating ones using rabbit antisera raised against a non-agglutinating strain as well as serotype I and II strains.   Pulsed-field gel electrophoresis (PFGE) was also conducted to clarify the genetic relationship among the strains.

Materials and Methods

Bacteria

A total of 188 strains of *S. parauberis* isolated from diseased Japanese flounder between 2002 and 2012 were used (Table 1).   They were identified as *S. parauberis* by species-specific PCR (Mata *et al*., 2004) and stocked at –80°C in Todd-Hewitt broth (TH; Difco) supplemented with 10% glycerol.   For preparation of formalin-killed cells (FKC), bacterial cells of each strain grown in TH broth or agar at 27°C for 24 h were inactivated with 0.5% formalin for 2 days, washed three times with sterilized phosphate-buffered saline, pH 7.2, (PBS) and resuspended in PBS at 100 mg/mL.   FKC suspension was added with 0.1% formalin as preserva-

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

Table 1. Source of *S. parauberis* strains used in this study

| Year | No. of strains from | | | | | | | Total |
	Kagawa	Shimane	Ehime	Oita	Kumamoto	Kagoshima	Nagasaki	
2002	11							11
2003	1			1				2
2004	3	1	2			3		9
2005	4		14	7			1	26
2006			1	6		2	2	11
2007	3		11	13				27
2008	2		12	10	1		9	34
2009			16	11			9	36
2010	2		7	8			10	27
2011							2	2
2012							3	3
Total	26	1	63	56	1	5	36	188

tive and stored at 4°C.

Antisera

Rabbit antisera were raised against FKCs of *S. parauberis* NUF1003 (serotype I) and NUF1071 (a non-agglutinating strain) according to the previous report (Kanai *et al*., 2009). The antisera against strains KRS02083 (serotype I) and KRS02109 (serotype II) prepared in the previous study (Kanai *et al.*, 2009) were also used. The strains KRS02083 and KRS02109 were isolated in Kagawa Prefecture in 2002, NUF1003 was isolated in Shimane Prefecture in 2004, and NUF1071 was isolated in Nagasaki Prefecture in 2008.

Microtiter agglutination test

A two-fold serial dilution of rabbit antisera in PBS was carried out in a 96-well microtiter plate. To 25 μL of serially diluted antisera, the same volume of FKC suspension (approximately 2 mg/mL in PBS) of each strain was added, and the mixture was agitated thoroughly and incubated at 4°C overnight. Agglutination titer was expressed by the reciprocal of the highest dilution of antiserum at which more than 90% of FKC agglutinated.

Preparation of absorbed antisera

For antiserum cross-absorption test, absorbed antisera were prepared by mixing each antiserum with the FKC (200 mg/mL of antiserum) of an absorbing strain and incubating at 4°C overnight. After centrifugation (13,000 ×*g*, 2 min), the supernatant was designated the absorbed antiserum. The absorption procedure was repeated until the agglutination titer against the absorbing FKC became < 4.

PFGE analysis

Bacterial cells grown on TH agar at 27°C overnight were washed twice and suspended at 4.5×10^9 CFU/mL in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Gel plugs were made by mixing 110 μL of the bacterial suspension with the same volume of 1.6% low melting-point agarose (Bio-Rad) in TE buffer using PFGE molds (Bio-Rad). Each solidified plug was placed into 380 μL of lysozyme solution (10 mM Tris-HCl, 50 mM NaCl, 5 mg/mL lysozyme, pH 7.2) and incubated at 37°C for 3 h with gentle shaking. After the lysozyme solution was discarded, 780 μL of proteinase K solution (0.1 M EDTA, pH 8.0, 1% sodium N-lauroyl sarcosinate, 0.5% SDS, 0.25 mg/mL proteinase K) was added and incubated at 50°C for 20 h. Plugs were washed three times in TE buffer supplemented with 1 mM phenylmethylsulfonyl fluoride for 1 h at room temperature and twice in 10 mM Tris-HCl (pH 8.0) at 4°C for 20 min with gentle shaking. *Sma*I (Takara), at a final concentration of 1 U/mL, was used for restriction endonuclease digestion at 30°C for 40 h according to the manufacturer's instructions. PFGE was performed in 1% agarose gel (Pulsed Field Certified Agarose, Bio-Rad) using a CHEF-DR III system (Bio-Rad) at 6 V/cm for 18 h at 14°C with pulse time switched from 0.5 s to 25 s under included angle 120°. The gel was stained with ethidium bromide for 30 min, destained in distilled water and photographed under UV light. The banding patterns were compared using ATTO Lane & Spot Analyzer ver 6.0 software. Dendrogram was constructed based on the unweighted pair group method with arithmetic averages (UPGMA) with MEGA ver 6.0 software.

Results

Serotyping of S. parauberis *strains*

From the agglutination titers of FKCs against four kinds of antiserum, 188 *S. parauberis* strains were divided into five groups; three subtypes of serotype I (Ia, Ib and Ic), serotype II and non-typeable (Table 2). Strains of subserotypes Ia and Ic were distinguished each other using anti-subserotype Ia (KRS02083) and Ic (NUF1071) sera, that is, subserotypes Ia and Ic showed high titers against anti-Ia and Ic sera,

Table 2.   Serotyping of *S. parauberis* based on agglutination titers of FKCs against rabbit antisera

Antiserum	Agglutination titer* of strains belong to				
	Subserotype Ia (n = 6)	Subserotype Ib (n = 91)	Subserotype Ic (n = 7)	Serotype II (n = 62)	Non-typeable (n = 22)
Anti-KRS02083 (Ia)	128–256	32–128	8–16	< 4	64– > 65,536
Anti-NUF1003 (Ib)	< 4–32	8–64	4–16	< 4–32	32–8,192
Anti-NUF1071 (Ic)	< 4	16–128	64–256	< 4	64–4,096
Anti-KRS02109 (II)	< 4	< 4	< 4	256–2,048	16–32,768

* Agglutination titer was expressed by the reciprocal of the highest dilution of antiserum at which more than 90% of FKC agglutinated.

Table 3.   Agglutination titers of representative serotype I strains belonged to three subserotypes against unabsorbed and absorbed antisera

Antiserum	Absorbed with FKC of	Agglutination titer[*1] of FKC of		
		KRS02083 (Ia)	NUF1003 (Ib)	NUF1071 (Ic)
Anti-KRS02083 (Type Ia)	Unabsorbed	128	32	4
	KRS02083 (3)[*2]	< 4	< 4	< 4
	NUF1003 (4)	16	< 4	< 4
	NUF1071 (2)	128	32	< 4
Anti-NUF1003 (Type Ib)	Unabsorbed	16	16	4
	KRS02083 (3)	< 4	< 4	< 4
	NUF1003 (2)	< 4	< 4	< 4
	NUF1071 (3)	< 4	4	< 4
Anti-NUF1071 (Type Ic)	Unabsorbed	< 4	64	128
	KRS02083 (2)	< 4	64	128
	NUF1003 (4)	< 4	< 4	4
	NUF1071 (3)	< 4	< 4	< 4

[*1] Agglutination titer was expressed by the reciprocal of the highest dilution of antiserum at which more than 90% of FKC agglutinated.
[*2] No. in the parentheses indicates no. of repetition of absorption procedure.

respectively.   Subserotype Ib strains agglutinated with these antisera showing titers at an equal level. Although the agglutination titers of anti-Ib (NUF1003) serum were low in comparison with the other antisera, serotype II strains reacted weakly with it.   Out of 104 serotype I strains, 87.5% were classified into subserotype Ib.   Non-typeable strains, which showed relatively high titers against anti-subserotype Ia (KRS02083) and serotype II (KRS02109) sera, accounted for 11.7% of the total strains (Table 2).

Antiserum cross-absorption test

The antiserum cross-absorption test demonstrated the relationship among subserotypes Ia, Ib and Ic more clearly (Table 3).   In this test it was shown that subserotypes Ia and Ic were completely separated, since cross-absorption between them did not lower the agglutination titers of anti-Ia and Ic sera.   On the other hand, subserotype Ib could possess surface-exposed antigens common to Ia and Ic, since the agglutination titers of these antisera were reduced after absorbed with Ib FKC.

PFGE analysis

PFGE analysis of *sma*I-digested genomic DNAs revealed that *S. parauberis* strains were divided into 40 pulsotypes and classified into three clusters according to the distance value (> 0.35) (Fig. 1).   By clustering the pulsotypes, subserotypes Ib and Ic belonged to the same group (cluster I) and were not separated each other.   Subserotype Ia and serotype II constructed two respective clusters (clusters II and III).   Pulsotypes S3 and S13, and S34, S36, S38 and S39 were the main types for subserotype Ib and serotype II, respectively. Non-typeable strains belonged to clusters I or III.

Discussion

In this paper we described three subtypes of serotype I (Ia, Ib and Ic) in *S. parauberis* derived from Japanese flounder.   Until now, serotype I has been thought to be a single entity, and anti-serotype I (anti-KRS02083) (designated Ia in this study) and II (anti-KRS02109) sera have been used for serodiagnosis of *S. parauberis* in Japan (Kanai *et al*., 2009).   Now, using anti-subserotype Ic serum as well as anti-subsero-
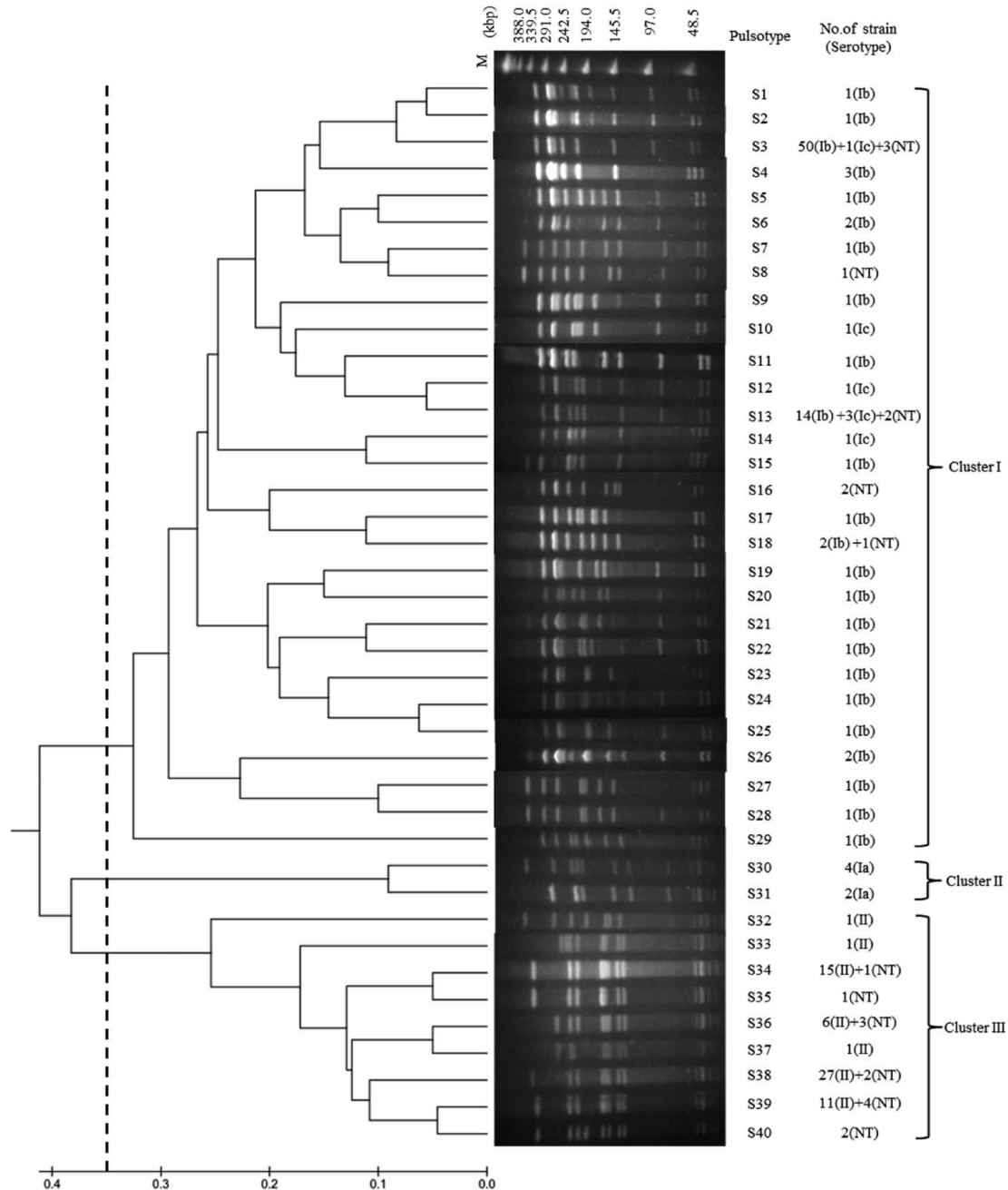
Fig. 1. Dendrogram of *S. parauberis* strains constructed by UPGMA cluster analysis of the PFGE patterns of *Sma*I-digested genomic DNA.   All the strains were classified into three clusters according to the distance value (> 0.35) indicated by the dotted line across the scale bar.   M: Lambda Ladder PFG Marker, NT: non-typeable (see text).

type Ia serum, strains that had been classified as serotype I could be distinguished to subserotypes Ia and Ib. Because serotype Ic has seldom been isolated, unidentifiable cases of *S. parauberis* would scarcely be encountered even if one uses only anti-serotype I (Ia) serum. However, upon serodiagnosis one should be aware of the existence of the subserotypes.

Although there are few investigations on the antigens that participate in the agglutination reaction of *S. parauberis* with antisera, Kanai *et al*. (2009) suggested that the capsular polysaccharides (CPS) were involved

in the serological variation of *S. parauberis*.   Therefore, differences in agglutination titers among the three subtypes of serotype I may come from the structural variation in CPS.   Kanai *et al*. (2009) also reported the serotype-specific precipitating reactions of autoclave-extracted cellular antigens of serotype I and II strains in an agar double-diffusion test, but the test could not distinguish between subserotypes Ia and Ib.   Thus subserotypes Ia and Ib may have a common CPS structure in part.   Similarly, the same thing would be said between subserotypes Ib and Ic, because subserotype Ib strains

agglutinate with anti-serotype Ic serum.　In other strep-tococci, CPS synthetic gene clusters were varied according to serotypes (Shibata *et al*., 2003; Mavroidi *et al*., 2007; Thurlow *et al*., 2009; Okura *et al*., 2013), indicating the relationship between CPS structure and serotypes.　However, although CPS is most likely involved in serotype specificity of *S. parauberis*, antigenic cell-surface proteins or other polysaccharides such as teichoic acids and rhamnopolysaccharides (Hancock and Gilmore, 2002) may also be involved.

PFGE analysis of the *S. parauberis* strains revealed the relationship between pulsotypes and serotypes.　This suggests that the serotypes are genetically separated.　However, subserotypes Ib and Ic were not separated in PFGE analysis.　As the reason for this, minor differences in genetic structure or expression of the CPS genes might exist between the two subserotypes.　Non-typeable strains were distributed to clusters I (subserotypes Ib and Ic) and III (serotype II). Kanai *et al*. (2009) found one non-typeable strain of *S. parauberis*, which agglutinated with both anti-serotype I and II sera but did not show precipitation reaction between its autoclave-extracts and the antisera, suggesting that the non-typeable strain was non-capsulated. The similar phenomena were observed in the KG⁺ phenotype of *Lactococcus garvieae* (Yoshida *et al*., 1997) and K⁻ phenotype of *S. iniae* (Kanai *et al*., 2006).　If the non-typeable strains were non-capsulated, their CPS synthetic gene(s) may be lost or mutated (Shutou *et al*., 2007; Morita *et al*., 2011).　Now, we are conducting studies to reveal the structure of CPS gene clusters of subserotypes Ia, Ib, and Ic, serotype II, and non-typeable strains.

Meng *et al*. (2009a) reported five serotype I strains that harbored an *ermB* gene on their chromosome and a *tet*(S) gene on an 11-kbp conjugative plasmid. These strains isolated in 2002 were originated from one prefecture.　In the present study, these strains and two additional strains isolated in 2007 and 2010 at another prefecture were revealed to be classified to subserotype Ia.　The latter two strains were shown to possess the 11-kbp plasmid, but did not harbor the *ermB* gene (data not shown).　Therefore, there is a risk of dissemination of subserotype Ia strains possessing the R-plasmid in the western part of Japan.

Serotypes of pathogens are a matter of concern not only for serodiagnosis but also for vaccine development.　At present, a commercially available vaccine for *S. parauberis* and *S. iniae* infections in Japanese flounder is used in aquaculture farms with high efficacy.　Our preliminary experiment showed a low protective effect in fish vaccinated with FKC of subserotype Ic followed by challenged with a virulent subserotype Ia strain, but a high protective effect was achieved in these fish challenged with serotype Ib or in fish vaccinated with FKC of subserotype Ib followed by challenged with the subserotype Ia strain.　These results suggest that a vaccine made from subserotype Ib cells would be effective against the infections caused by the three subtypes of serotype I, because subserotype Ib cells possess common cell-surface antigens among them.

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