RESEARCH LETTER

Phenotypic characteristics of *Streptococcus iniae* and *Streptococcus parauberis* isolated from olive flounder (*Paralichthys olivaceus*)

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Abstract

The etiological agents of streptococcosis were isolated from diseased olive flounder collected on the Jeju island of Korea. A total of 151 bacterial isolates were collected between 2003 and 2006. The isolates were examined using various phenotypic and proteomic analyses, including sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting, and glycoprotein assays. In addition, isolates were grown on blood agar to assess hemolytic activity, and biochemical assays were performed using the API20 Strep kit. Our results revealed that all isolates were nonmotile, Gram-positive cocci that displayed negative catalase and oxidase activities. Multiplex PCR assays revealed that 43% and 57% of the isolates were *Streptococcus iniae* and *Streptococcus parauberis*, respectively. These results were consistent with those of the SDS-PAGE and immunoblot analyses using whole-cell lysates of bacterial isolates. Significant differences were observed with respect to the Voges–Proskauer, pyrrodonyl arylamidase, alkaline phosphatase, and hemolytic activities of the *S. iniae* and *S. parauberis* isolates. Isolates of *S. iniae* displayed uniform profiles in the immunoblot and glycoprotein assays; however, immunoblot assays of *S. parauberis* isolates (using a chicken IgY antibody raised against a homologous isolate) revealed three distinct antigenic profiles. Our findings suggest that *S. parauberis* and *S. iniae* are endemic pathogens responsible for the development of streptococcosis in olive flounder.

Introduction

Streptococcosis of cultured fish contributes to major economic losses in the aquaculture industries of many countries, including Israel (Eldar *et al.*, 1995), Italy (Ghittino & Prearo, 1992), Japan (Kitao, 1993), Korea (Baeck *et al.*, 2006; Shin *et al.*, 2006) and the United States (Perera *et al.*, 1994). The major species responsible for this disease include *Streptococcus parauberis*, *Streptococcus iniae*, *Streptococcus difficilis*, *Lactococcus garvieae*, *Lactococcus piscium*, *Vagococcus salmoninarum*, and *Carnobacterium piscicola* (Mata *et al.*, 2004). In particular, *S. iniae* and *L. garvieae* are known to infect many fish species, including olive flounder (*Paralichthys olivaceus*) (Nakatsugawa, 1983; Shin *et al.*, 2006), rainbow trout (*Oncorhynchus mykiss*) (Chang *et al.*, 2002; Diler *et al.*, 2002), red drum (*Sciaenops ocellatus*) (Eldar *et al.*, 1999), rabbitfish (*Siganus canaliculatus*) (Yuasa *et al.*, 1999), yellowtail (*Seriola quinqueradiata*) (Kusuda *et al.*, 1976), and barramundi (*Lates calcarifera*) (Bromage *et al.*, 1999; Creeper & Buller, 2006). Recently, *S. parauberis* was determined to be the etiological agent of streptococcosis in turbot and olive flounder (Doménech *et al.*, 1996; Mata *et al.*, 2004; Baeck *et al.*, 2006).

Unfortunately, conventional biochemical tests do not allow for the precise identification and classification of streptococcal isolates, because of differences in growth rates, inoculum levels, and incubation periods (Facklam & Elliott, 1995). Consequently, the number and the nature of bacterial

species associated with fish streptococcosis remains controversial (Romalde *et al*., 2008). Recently, Shin *et al*. (2006) used molecular and serological tests to identify several distinguishing characteristics of *S. iniae* and *L. garvieae*. In addition, Baeck *et al*. (2006) used biochemical and serological methods (e.g. slide agglutination, hemolytic tests, antimicrobial susceptibility tests, and multiplex PCR analyses) to characterize *S. parauberis* isolates from diseased olive flounder. However, few studies have explored the phenotypic and serological characteristics of these pathogens.

Here, we use biochemical, serological, molecular, and traditional microbiological techniques to identify and characterize the streptococcal strains isolated from diseased olive flounder. Our findings will assist with future epidemiological studies and will promote the development of appropriate vaccines against streptococcosis.

Materials and methods

Collection of bacterial isolates

A total of 151 bacterial strains were isolated from the spleens of diseased olive flounder, which were obtained from aquaculture farms on the Jeju island of Korea between 2003 and 2006. Bacterial isolates were cultured on tryptic soy agar (TSA, Oxoid) at 25 °C for 24 h. Individual colonies were recultured on TSA to ensure purity. The isolates were then identified according to their source of collection (Table 1) and stored at − 70 °C, in tryptic soy broth (TSB) containing 10% (v/v) glycerol, until further use.

Bacterial cultures and biochemical test

Bacterial cultures and biochemical tests were performed as described previously (Shin *et al*., 2006). Briefly, stored isolates were inoculated in TSB, incubated at 25 °C for 24 h, and cultured on blood agar at 25 °C for 24 h. Colonies from the blood agar plates were subcultured in TSB and grown to an $OD_{610\,nm}$ of 1.0 (i.e. *c*. 4×10^9 CFU mL^{-1}) for use in PCR, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblot assays. In addition, colonies were suspended in phosphate-buffered saline (PBS) (3 mM KCl, 137 mM NaCl, 1.5 mM KH_2PO_4, and 8 mM Na_2HPO_4, pH 7.4) for catalase, oxidase, and motility tests. Other biochemical tests were performed using the API 20 Strep kit (BioMerieux Inc.) according to the manufacturer's instructions. Bacterial motility test was performed by the wet mount method using Nikon Eclipse TE2000-S microscope. Reference strains [*S. iniae* ATCC29178, *S. parauberis* (Dong-bo), and *L. garvieae* KG9408] were used as positive controls for comparing the results.

Multiplex PCR assays

Multiplex PCR assays were used to simultaneously detect *S. iniae*, *S. parauberis*, and *L. garviae*. The stored isolates were cultured in TSB at 25 °C for 24 h, and DNA was extracted using the AccuPrep® Genomic DNA Extraction Kit (Bioneer, Korea) according to the manufacturer's instructions. The oligonucleotide primer sets used to identify isolates were derived from published sequences (Table 2). Multiplex PCR was performed in 20-μL reaction mixtures containing 1 μL template DNA, 0.05 μM of each primer (Bioneer), and the AccuPower PCR® premix (Bioneer). Amplification was performed using a PTC-100TM programmable thermal controller (MJ Research Inc.). The PCR conditions included an initial denaturation cycle of 94 °C for 5 min; 30 serial cycles that each included a denaturation step at 94 °C for 30 s, an annealing step at 50 °C for 30 s, and an extension step at 72 °C for 30 s; and a final extension step at 72 °C for 7 min. Multiplex PCR products were analyzed on a 2% (w/v) agarose gel containing 1% (w/v) ethidium bromide. DNA bands were visualized under UV transillumination and photographed.

Production of chicken anti-*S. parauberis* (Dong-bo) IgY

Anti-*S. parauberis* (Dong-bo) IgY antibodies were raised in a chicken as described previously (Shin *et al*., 2006). The

Table 1. Origins of bacterial isolates

		Number of isolates per month												
Years	Species	January	February	March	April	May	June	July	August	September	October	November	December	Total
2003	SI	1	1	0	0	0	2	4	5	3	2	3	2	23
	SP	4	2	3	2	2	3	2	2	1	2	1	3	27
2004	SI	1	1	0	1	1	2	2	2	0	2	2	1	15
	SP	1	1	4	1	2	2	2	1	0	1	2	2	19
2005	SI	1	0	0	0	2	1	1	1	2	2	1	2	13
	SP	2	2	2	1	1	2	1	2	2	2	2	1	20
2006	SI	1	0	1	1	1	1	2	2	2	1	1	1	14
	SP	1	1	1	2	3	1	0	5	2	2	1	1	20
	Total	12	8	11	8	12	14	14	20	12	14	13	13	151

SI, *Streptococcus iniae*; SP, *Streptococcus parauberis*.

Table 2. Multiplex PCR primers

Pathogen	Primer	Nucleotide sequence (5′–3′)	Expected size (bp)	References
S. iniae	Sin 1b	CTAGAGTACACATGTAGCTAAG	300	Zlotkin *et al*. (1998)
	Sin 2	GGATTTTCCACTCCCATTAC		
S. parauberis	Spa 2152	TTTCGTCTGAGGCAATGTTG	718	Mata *et al*. (2004)
	Spa 2870	GCTTCATATATCGCTATACT		
L. garvieae	pLG 1	CATAACAATGAGAATCGC	1100	Mata *et al*. (2004)
	pLG 2	GCACCCTCGCGGGTTG		

advantage of using IgY is that a large amount of specific antibodies can be produced without sacrificing experimental animals. Briefly, chickens were immunized with 10^8 CFU mL^{-1} of formalin-killed bacteria (FKB, the Dong-bo isolate of *S. parauberis*) that had been mixed with an equal volume of Freund's complete adjuvant. The chickens were then injected every 2 weeks, for a total of three injections, with 10^8 CFU mL^{-1} of FKB emulsified with Freund's incomplete adjuvant. A week after the final immunization, chicken eggs were collected and IgY was purified using the EggSTRACT® IgY purification kit (Promega, Madison, WI) according to the manufacturer's instructions. The purified IgY pellet was resuspended in PBS and stored at −20 °C until further use.

SDS-PAGE

SDS-PAGE analyses were performed on 12.5% (w/v) separating gels, according to the method of Laemmli (1970). Bacterial isolates were cultured in TSB at 25 °C for 24 h, centrifuged at 2000 ***g*** for 30 min, washed three times in PBS, resuspended in 200 μL of PBS, and mixed with 50 μL of 5 × sample buffer [5 : 1; 60 mM Tris-HCl, 25% (v/v) glycerol, 2% (w/v) SDS, 14.4 mM 2-mercaptoethanol, and 0.1% (w/v) bromophenol blue]. The samples were then sonicated 10 times (XL-2020, Misonix Inc., Farmingdale, NY) (5.5 W, 10 s intervals), boiled for 10 min, cooled on ice, and centrifuged at 16 000 ***g*** for 20 min at 4 °C. The supernatants were collected and stored at −20 °C until further use. After SDS-PAGE analysis, gels were stained with Coomassie Brilliant Blue R-250. Additional SDS-PAGE gels were run for use in Western blotting assays.

Immunoblot assays

After proteins were resolved via SDS-PAGE, gels were electroblotted onto polyvinylidene difluoride (PVDF) membranes (70 V for 70 min). The membranes were then soaked in 100% methanol for 20 s and dried at room temperature (RT). The membranes were blocked with 5% (w/v) skim milk in PBS-T [3 mM KCl, 137 mM NaCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4 (pH 7.2), and 0.05% (v/v) Tween-20] for 60 min at RT, washed three times with PBS-T, and incubated with anti-*S. parauberis* (Dong-bo) chicken IgY (1 : 200) for 60 min at RT. Membranes were then washed three times with PBS-T and incubated with rabbit anti-chicken IgG-HRP (1 : 4000) (Cappel Biologicals) for 90 min at RT. After three washes with PBS-T for 15 min each, the membrane was developed using the enhanced chemiluminescence kit (Amersham Biosciences) and exposed to an X-ray film to allow visualization of antigenic proteins.

Glycoprotein detection assays

In preparation for glycoprotein assays, bacterial proteins were resolved on SDS-PAGE and electroblotted onto a PVDF membrane as described above. Glycoproteins were detected using an immunoblot kit from Bio-Rad Laboratories (catalog no. 170-6490) according to the manufacturer's instructions. The membranes were washed with PBS (9 mM NaH_2PO_4, 27 mM NaCl, pH 7.2) for 10 min at RT, and immersed in sodium acetate/EDTA buffer containing sodium periodate (100 mM sodium acetate, 5 mM EDTA, 10 mM sodium periodate, pH 5.5) and incubated in the dark for 20 min at RT. The membranes were then washed three times with PBS, for 10 min each. A biotinylation solution was prepared immediately before use, by adding 2 μL hydrazide solution to 10 mL sodium acetate/EDTA buffer (without sodium periodate) and the membranes were immersed in this solution for 60 min at RT. The membranes were then washed three times, 10 min each at RT, with Tris-buffered saline (TBS: 50 mM Tris base, 27 mM NaCl, pH 7.2) and incubated for 30 min at RT with blocking solution (dissolve reagent 0.5 g provided product in 100 mL TBS) and washed three times with TBS for 10 min each at RT. Streptavidin–alkaline phosphatase conjugate was prepared and the membranes were incubated in this solution for 60 min at RT, and were again washed with TBS. Gentle agitation was applied to the membranes throughout the procedure, except during the development of the reaction. The developer was prepared immediately before use by mixing together nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, according to manufacture's specifications. The membranes were incubated in this solution at RT until the required reaction intensity was achieved.

The reaction was stopped by rinsing the membrane several times in ddH_2O.

Experimental challenges

Duplicate groups of fish ($n = 15$ per group) were intraperitoneally injected with 4×10^8 CFU of randomly selected *S. iniae* and *S. parauberis* isolates (or a TSB control). Olive flounder (20 ± 2 cm in length) were purchased from a commercial aquaculture farm, and were determined to be free of streptococcal infection by conducting the above-mentioned microbiological tests and multiplex PCR analysis. The challenge room was maintained at 24 ± 1 °C. Fish were maintained in 200-L fiberglass-reinforced plastic aquaria supplied with flow-through seawater, and were subjected to 12-h light/dark cycles. Fish were monitored daily (for a total of 15 days after challenge) for clinical signs of disease and mortality. Moribund and dead fish were removed twice daily, and bacterial samples were cultured at 25 °C for 24 h on sheep blood agar obtained from head kidneys. Isolates were identified using multiplex PCR, as described above.

Results

Identification of the streptococcal agents of olive flounder

Bacterial isolates were collected from olive flounder exhibiting clinical signs of streptococcosis. General microbiological examinations revealed that the isolates were nonmotile, Gram-positive cocci, with no catalase or oxidase activities. Hemolytic analysis showed that 65 isolates were β-hemolytic, whereas 86 isolates were α-hemolytic. The β-hemolytic and α-hemolytic isolates were identified as *S. iniae* and *S. parauberis*, respectively, via multiplex PCR (Fig. 1).

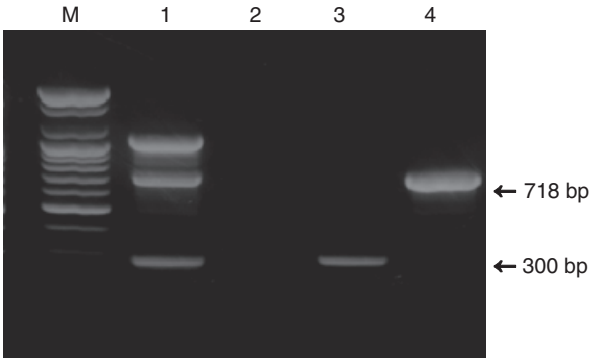

Fig. 1. Multiplex PCR assays of *Streptococcus iniae* and *Streptococcus parauberis*. Lanes: M, 100 bp DNA ladder; 1, mixed template (DNA from *S. iniae*, *S. parauberis*, and *Lactococcus garvieae*); 2, negative control; 3, group A (*S. iniae*); 4, group B (*S. parauberis*).

Biochemical characterization of streptococcal isolates

Biochemical analyses revealed consistent patterns in most *S. iniae* isolates (e.g. positive reactions on the PYRA, PAL, and LAP tests and negative reactions on the VP, HIP, ESC αGAL, βGUR, βGAL, ARA, SOR, LAC, INU, and RAF tests). In addition, variable reactions were observed on the ADH, RIB, MAN, TRE, AMD, and GLYG tests. Most *S. parauberis* isolates displayed positive reactions to VP, LAP, and TRE and negative reactions to αGAL, βGUR, βGAL, PAL, ARA, RAF, AMD, and GLYG. However, *S. parauberis* isolates displayed variable reactions to HIP, ESC, PYRA, ADH, RIB, MAN, SOR, LAC, and INU (Table 3).

SDS-PAGE and immunoblot profiling

We performed SDS-PAGE and immunoblot assays to compare the proteomic and antigenic profiles of the two types of

Table 3. Biochemical characteristics of *Streptococcus iniae* and *Streptococcus parauberis* from diseased olive flounder using olive flounder*

	S. iniae†	*S. iniae* ATCC29178 Buller (2004)	*S. parauberis*	*S. parauberis* (Dong-bo)
Cat	0 (0%)	−	0 (0%)	−
Oxi	0 (0%)	−	0 (0%)	−
Hem	β (100%)	β	α (100%)	α
VP	0 (0%)	−	86 (100%)	+
HIP	0 (0%)	−	59 (68.6%)	+
ESC	2 (3.1%)	+	65 (80%)	+
PYRA	65 (100%)	+	6 (6.9%)	−
αGAL	0 (0%)	−	0 (0%)	−
βGUR	1 (1.5%)	−	0 (0%)	−
βGAL	0 (0%)	−	0 (0%)	−
PAL	65 (100%)	+	1 (1.2%)	+
LAP	65 (100%)	+	86 (100%)	+
ADH	7 (10.8%)	+	44 (51.2%)	+
RIB	50 (76.9%)	−	40 (46.5%)	+
ARA	0 (0%)	−	1 (1.2%)	−
MAN	45 (69.2%)	−	75 (87.2%)	+
SOR	0 (0%)	−	58 (67.4%)	+
LAC	0 (0%)	−	27 (31.3%)	−
TRE	62 (95.4%)	+	86 (100%)	+
INU	0 (0%)	−	52 (60.5%)	+
RAF	0 (0%)	−	1 (1.2%)	−
AMD	48 (73.8%)	+	0 (0%)	−
GLYG	37 (56.9%)	+	1 (1.2%)	−

*Following analysis using API 20 strep kits, isolates were identified as either *Streptococcus iniae* or *Streptococcus parauberis* by multiplex PCR.
†Percent positive reactions are shown in parentheses.
Cat, catalase; Oxi, oxidase; Hem, hemolysis; VP, Voges–Proskauer; HIP, hipuric acid; ESC, esculin; PYRA, pyrrolidonyl arylamidase; αGAL, α-galactosidase; βGUR, β-glucuronidase; βGAL, β-galactosidase; PAL, alkaline phosphatase; LAP, leucine aminopeptidase; ADH, arginine dihydrolase; RIB, ribose; ARA, arabinose; MAN, mannitol; SOR, sorbitol; LAC, lactose; TRE, trehalose; INU, inulin; RAF, rafinose; AMD, amidon; GLYG, glycogen; −, negative reaction; +, positive reaction.

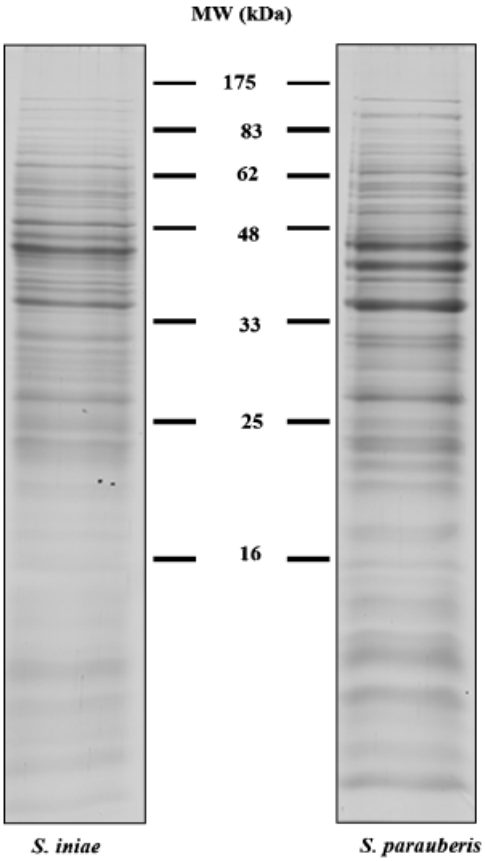

Fig. 2. SDS-PAGE analysis of whole-cell lysates from *Streptococcus iniae* and *Streptococcus parauberis*.

streptococcal isolates (Fig. 2). The SDS-PAGE profiles of the *S. iniae* isolates consisted of eight major bands, with approximate molecular weights (MWs) of 65, 60, 48, 46, 44, 35, 32, and 26 kDa. The *S. parauberis* isolates exhibited five major bands, with approximate MWs of 46, 43, 35, 26, and 23 kDa.

Immunoblot assays using anti-*S. parauberis* (Dong-bo) IgY antibody revealed a uniform antigenic pattern for *S. iniae* isolates (antigens with MWs of 83, 78, 53, 50, 46, 33, and 25 kDa were identified) (Fig. 3, lane 1). However, *S. parauberis* isolates exhibited three distinct antigenic profiles, in which major common antigens of 83, 52, 46, 33, and 25 kDa were identified (Fig. 3, lanes 2, 3 and 4). The *S. parauberis* isolates could be separated into three types, according to the presence of additional antigenic bands. The first pattern consisted of additional antigens with MWs of 72, 65, 42, and 22 kDa, whereas the second pattern consisted of additional antigens with MWs of 78, 40, 28, and 23 kDa. The third pattern consisted of additional antigens with MWs of 78, 55, and 42 kDa (Fig. 3, lanes 2, 3 and 4). The first, second, and third antigenic protein patterns were observed in 67, 18, and 1 isolate of *S. parauberis*, respectively.

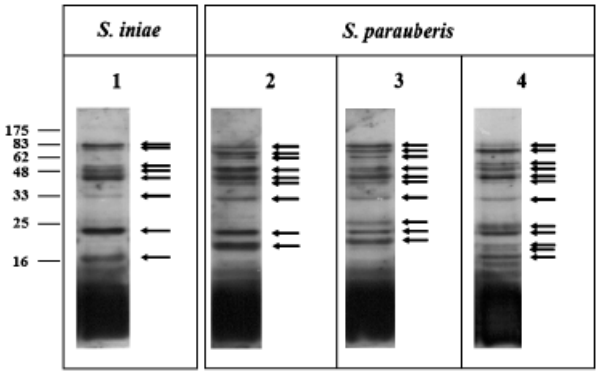

Fig. 3. Immunoblot assays of whole-cell lysates from *Streptococcus parauberis* and *Streptococcus iniae*, using chicken anti-*S. parauberis* (Dong-bo) IgY antibody. Lanes: 1, antigenic profiles of *S. iniae* (65 isolates); 2, antigenic profiles of *S. parauberis* (67 isolates); 3, antigenic profiles of *S. parauberis* (18 isolates); 4, antigenic profile of *S. parauberis* (one isolate).

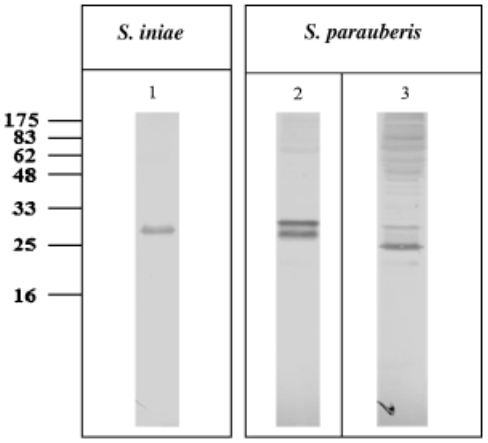

Fig. 4. Glycoprotein analysis of whole-cell lysates of *Streptococcus iniae* and *Streptococcus parauberis*. Lanes: 1, *S. iniae* (65 isolates); 2, *S. parauberis* (85 isolates); 3, *S. parauberis* (one isolate).

Glycoprotein detection assays

The glycoproteins of *S. iniae* and *S. parauberis* were examined for differences in their carbohydrate structures (Fig. 4). The glycoprotein profile of *S. iniae* was uniform, with a unique band of 28 kDa (Fig. 4, lane 1). With the exception of one isolate, all *S. parauberis* isolates exhibited two major glycoprotein bands of 26 and 30 kDa (Fig. 4, lane 2). The remaining *S. parauberis* isolate exhibited three major glycoprotein bands of 20, 25, and 28 kDa (Fig. 4, lane 3).

Experimental challenge

Mortality in the fish injected with *S. iniae* started after day 4 of the challenge, with none surviving past the sixth day. Fish

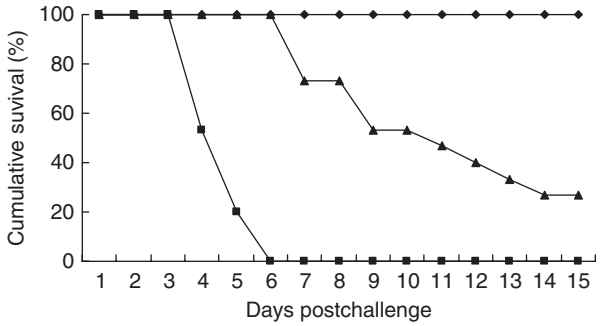

Fig. 5. Survival of olive flounder (%) after artificial infection with a TSB control (◆), 4×10^9 cells of *Streptococcus iniae* (■) or 4×10^9 cells of *Streptococcus parauberis* (▲).

injected with *S. parauberis* began to die 7 days after the challenge, with 26% survival on the final day of the experiment. No fish died in control groups (Fig. 5). The clinical signs of *S. iniae* infection included hemorrhagic septicemia, enterocele, exophthalmia, and erratic swimming. The clinical signs of *S. parauberis* infection included darkened body coloration and cachexia.

Microbiological analysis of the bacterial isolates collected from moribund or dead fish was positive for their respective groups, i.e. β-hemolytic for the *S. iniae*-infected group and nonhemolytic for the *S. parauberis* group. These isolates were further confirmed as *S. iniae* and *S. parauberis*, respectively, by multiplex PCR analysis.

Discussion

Streptococcosis is associated with acute and chronic mortality in many aquaculture species. In this study, we obtained bacterial isolates from olive flounder exhibiting clinical symptoms of hemorrhagic septicemia, exophthalmia, and meningitis. All bacterial isolates ($n = 151$) were nonmotile, Gram-positive cocci, with no oxidase or catalase activities. Similar symptoms and clinical signs are associated with warm-water streptococcosis (Doménech *et al.*, 1996; Eldar *et al.*, 1999) caused by *L. garviae*, *S. parauberis*, *S. iniae*, and *S. difficilis* (Muzquiz *et al.*, 1999).

Phenotypic and biochemical characterizations (i.e. growth on blood agar and analysis using the API20 Strep kit, respectively) revealed the presence of two streptococcal species in infected olive flounder. However, the biochemical results obtained from the API 20 Strep kit were not consistent with those reported for *S. parauberis* (Baeck *et al.*, 2006) and *S. iniae* (Shin *et al.*, 2006). These variations may reflect differences in the number of isolates tested, geographical locations, or physiochemical factors (e.g. temperature and salinity). On the other hand, RNA sequencing is a powerful method for analyzing phylogenic interrelationship in *Streptococcus* species. The 16S rRNA gene sequence of *S. iniae* and *S. parauberis* is reported to have 98.1% homology (Bentley *et al.*, 1991). However, the high homology was suspected to be due to the difference in the pathogenicity: *S. iniae* showed acute mortality, but *S. parauberis* demonstrated a chronic pattern. Further investigation on the relationships between *S. iniae* and *S. parauberis* by rRNA might be needed with the isolates. The inconsistencies in these biochemical tests and rRNA analysis reveal the inadequacies of currently appropriate identification methods. In the present situation, analysis via multiplex PCR revealed that 43% (65 of 151) and 57% (86 of 151) of the bacterial isolates were *S. iniae* and *S. parauberis*, respectively. These results are consistent with previous reports (Baeck *et al.*, 2006), suggesting that *S. parauberis* may be the dominant strain responsible for streptococcosis of olive flounder in Korea.

Analysis of whole-cell protein profiles by SDS-PAGE has been suggested to be an efficient tool for differentiating various streptococcal species (Vandamme *et al.*, 1998). Shin *et al.* (2006) reported several differences in the SDS-PAGE protein profiles of *S. iniae* and *L. garvieae* isolates from olive flounder. Similarly, in the present study, we observed differences in the whole-cell protein profiles of *S. iniae* and *S. parauberis* isolates. Immunoblot assays were performed to compare the antigenic protein profiles of whole-cell bacterial lysates using anti-*S. parauberis* (Dong-bo) chicken IgY. This assay provides useful information on similarities and differences between various bacterial isolates (Schade *et al.*, 2000), and will assist in the development of an effective vaccine. The results of our immunoblot assays revealed several common and species-specific antigenic bands, which were used to distinguish the *S. iniae* and *S. parauberis* isolates. However, *S. parauberis* isolates exhibited three distinct antigenic patterns (Fig. 3). This serological heterogeneity may reflect differences in the virulence of the isolates. We are currently investigating possible virulence differences between serologically heterogeneous *S. parauberis* isolates, to assist in the development of an effective vaccine.

Glycoproteins vary considerably between bacterial species and protect against cell death (mediated either by attack from normal and immune sera or by phagocytosis). In addition, glycoproteins mediate attachment to host cells (Hansmeier *et al.*, 2004). We observed several distinct differences in the glycoprotein patterns of *S. iniae* and *S. parauberis*. Variations in glycoproteins may reflect differences in the pathogenic characteristics of *S. iniae* (which causes an acute infection) and *S. parauberis* (which causes a chronic infection). However, we cannot presently confirm this hypothesis.

The experimental challenge revealed that *S. iniae* and *S. parauberis* differ with respect to virulence and clinical signs. Infection with *S. iniae* proceeds faster in olive flounder

than does infection with *S. parauberis*. In addition, *S. iniae* induces the typical clinical signs of streptococcosis (Roberts, 2001), whereas *S. parauberis* infections manifest as darkened body surfaces and cachexia. We propose that the pathogenic variations reflect differences in virulence factors and pathogenic mechanisms used by the bacteria; such differences have not been examined in previous studies. Thus, further studies should investigate virulence factors and pathogenic mechanisms used by streptococcal species.

Here, we characterized *S. iniae* and *S. parauberis* isolates from diseased olive flounder using microbiological and biochemical tests, multiplex PCR, SDS-PAGE, immunoblot analysis, and glycoprotein assays. Our results strongly indicate that *S. parauberis* is an emerging pathogen causing streptococcosis in cultured olive flounder in Korea. Further examination of the virulence of serologically heterogeneous *S. parauberis* isolates is currently underway, in an effort to develop effective vaccines against streptococcosis in olive flounder.

Acknowledgement

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