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Role of Capsule in Immunogenicity of *Streptococcus iniae* to Japanese Flounder *Paralichthys olivaceus*

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ABSTRACT—*Streptococcus iniae* causes an acute systemic disease in Japanese flounder *Paralichthys olivaceus*, one of the important cultured fish in Japan. Although commercial *S. iniae* vaccines have been developed and used in farms since 2005, the immunoprotection mechanism in vaccinated fish has not been elucidated yet. To verify the involvement of capsular polysaccharides of *S. iniae* in immunoprotection, we compared the protective efficacy of formalin-killed cells (FKC) of *S. iniae* NUF631 (capsulated) with those of its isogenic capsular-deleted mutants in *S. iniae* infection of Japanese flounder. As a result, high protection was achieved by immunization with NUF631 FKC but not with mutant FKC. Viable count of intravenously inoculated NUF631 decreased in the kidney of flounder immunized with NUF631 FKC, and NUF631 cells opsonized with anti-NUF631 flounder serum elevated the phagocytic activity and reactive oxygen species production of flounder peritoneal macrophages. In electron microscopic examination, electronically dense materials were observed on the capsule of NUF631 cells pretreated with the antiserum, and in western blot analysis flounder antibody was detected in the antiserum components bound to NUF631 cells. These findings indicate that capsular polysaccharides are important protective antigens and anti-capsule antibody plays a protective role as an opsonin in flounder *S. iniae* infection.

Key words: capsule, capsule-deleted mutant, immunoprotection, *Streptococcus iniae*, *Paralichthys olivaceus*, Japanese flounder

Japanese flounder *Paralichthys olivaceus*, one of the important marine fish species cultured in Japan, has frequently been suffered from streptococcicosis caused by *Streptococcus iniae* in high temperature months (Nakatsugawa, 1983). Control of the streptococcal infection in Japanese flounder cultivation had depended on antibiotic chemotherapy with tetracycline untill the year 2005 when a commercial *S. iniae* vaccine became available. However, neither defense mechanisms of vaccinated fish nor protective antigens have not been clarified yet. Kanai *et al.* (2006) reported the presence and properties of two serological phenotypes, designated K^+ type and K^- type, in Japanese *S. iniae* isolates. They showed that strains of K^+ type possessed a type-specific antigen, cell surface acidic polysaccharides and a capsule, and that vaccination with formalin-killed cells (FKC) of K^+ type, but not with K^- type FKC, confers high protective immunity on Japanese flounder against *S. iniae* infection. In the previous paper (Shutou *et al*., 2007), using isogenic capsular polysaccharide-deleted mutants of K^+ type we have shown that the K^+ type-specific antigen of *S. iniae* is the cell surface acidic polysaccharides and a component of the capsule. It is known that capsular polysaccharides are the major immunogenic components in a number of vaccines, for example, pneumococcal, staphylococcal, meningococcal and group B streptococcal vaccines (Artz *et al*., 2003; O'Riordan and Lee, 2004; Lindahl *et al*., 2005; Harrison, 2006). So, it is speculated that the capsular polysaccharides of *S. iniae* are one of the protective antigens.

In this study, we evaluated protective efficacies of FKC of *S. iniae* capsulated and isogenic capsule-deleted strains in Japanese flounder and investigated immunoprotection mechanisms in vaccinated fish to *S. iniae* infection.

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Materials and Methods

Bacteria

S. iniae NUF631 (K^+ type) and the isogenic capsular polysaccharide-deleted mutant strains 4−58, 4−79, 4−94, 6−20, 10−15, 10−86, 11−34 and 11−36 (Shutou *et al*., 2007) were used in this study. They were routinely cultured using Todd-Hewitt agar or broth (Difco Laboratories) for 18−24 h at 27°C. FKC of each strain was prepared as described previously (Kanai *et al*., 2006).

Vaccination test

Nine groups of 15 Japanese flounder, average body weight of 127.2 g, were vaccinated intraperitoneally with FKC (2 mg/mL) of *S. iniae* NUF631 or mutant strains at 0.5 mL/100 g body weight. As a control, 15 fish were inoculated with phosphate-buffered saline (PBS). At 2 wk post-vaccination, ten fish from each group were challenged by intraperitoneal injection with *S. iniae* NUF631 at a dose of 5.3×10^5 CFU/100 g body weight, and the mortalities were monitored for 2 wk. Blood was collected from the residual five fish of each group and serum was separated from the blood by centrifugation at $900 \times g$ for 15 min. Agglutination titers of the sera were measured with FKC of *S. iniae* NUF631 and NUF44 (K^- type) according to the previous paper (Kanai *et al*., 2006). Average water temperature during the vaccination period and after challenge was 25.5°C and 26.3°C, respectively.

Fate of S. iniae *NUF631 in immunized flounder*

Two groups of nine flounder, average body weight of 418.8 g, were vaccinated intraperitoneally with FKC (2 mg/mL) of *S. iniae* NUF631 or 6−20 at 0.5 mL/100 g body weight. As a control, nine fish were inoculated with PBS. At 2 wk post-vaccination, the fish were inoculated intravenously with *S. iniae* NUF631 cell suspension (10^6 CFU/mL) at 0.1 mL/100 g body weight. Viable counts of *S. iniae* in the kidney and blood were monitored for 3 days as described previously (Shutou *et al*., 2007). Average water temperature during the immunization period and after challenge was 26.1°C and 26.0°C, respectively.

Viability of S. iniae *NUF631 in plasma of immunized flounder in vitro*

Blood was collected with a heparinized syringe from flounder immunized with NUF631 or 6−20 FKC, and plasma was separated by centrifugation at $900 \times g$ for 15 min. Changes in viable count of NUF631 in the plasma were measured as described previously (Shutou *et al*., 2007).

Preparation of flounder immune sera

Flounder immune sera were raised by immunizing 1-year-old flounder with NUF631 FKC or 6−20 FKC for 2 wk and stocked at −30°C. They were designated anti-NUF631 serum and anti-6−20 serum, respectively.

Phagocytic activities of flounder peritoneal macrophages

Preparation of flounder peritoneal macrophages, phagocytosis assay and chemiluminescence assay were conducted as described previously (Shutou *et al*., 2007). *S. iniae* NUF631 and 6−20 cells were opsonized with normal or immune flounder sera for 20 min at 27°C before each assay was performed.

Transmission electron microscopy (TEM) of cell surface of S. iniae

Preparation of samples for TEM was performed as described by Kanai *et al*. (2006). *S. iniae* NUF631 and 6−20 cells were pretreated with an equal volume of normal or immune flounder sera.

Separation of serum components bound to cell surface of S. iniae

One hundred microliters of NUF631 or 6−20 cell suspension (100 mg/mL) and 400 μL of normal or immune flounder sera were mixed, incubated for 30 min at 27°C and cooled on ice. The bacterial cells were washed with PBS to remove unattached serum components and suspended in 80 μL of 1% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]propanesulfonic acid) · PBS to detach bound serum components from the cells for 10 min at 27°C. Supernatants were collected by centrifugation.

Detection of flounder antibody by western blotting

The supernatants containing serum components bound to the bacterial cells were dialyzed to distilled water for 24 h at 4°C and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide separating gel with 4% stacking gel (Laemmli, 1970). After electrophoresis, the gel was equilibrated with transfer buffer (100 mM Tris, 192 mM glycine, 20% methanol), and proteins in the gel were transferred electrophoretically to PVDF membrane (Atto). After blocking nonspecific binding sites with 3% gelatin, the membrane was incubated for 2 h in rabbit antiserum against flounder antibody diluted 1:400 with 1% gelatin TTBS (20 mM Tris, 500 mM NaCl, pH 7.5, 0.05% Tween20). The membrane was washed three times with TTBS and incubated for 2 h in goat anti-rabbit IgG-HRP conjugate (Bio-Rad) diluted 1:3,000 with 1% gelatin TTBS. The membrane was washed three times with TTBS and soaked into DAB (3, 3'-diaminobenzidine tetrahydrochloride) solution (10 mg/50 mL in 50 mM Tris-HCl, pH 7.5) containing 0.005% H_2O_2 for 15 min.

Statistics

Statistical analysis of protective efficacy of the vaccines was performed by Fisher's exact test. Signific-

ance of difference in bacterial clearance test, phagocytosis assay and chemiluminescence assay were analysed using student's *t*-test.

Results

Efficacy of vaccines

Mortalities of fish immunized with mutant FKC were significantly higher than that of NUF631 FKC. All dead fish exhibited signs of the streptococcicosis, and *S. iniae* was reisolated from them. *S. iniae* was also isolated from survived fish immunized with mutant FKC but not from those immunized with NUF631 FKC.

Table 1. Efficacy of FKC vaccines in challenge infection with *S. iniae* NUF631

S. iniae strain	No. of fish died/ No. of fish used	Agglutination titer	
		Anti-K^- type	Anti-K^+ type
NUF631	2/10	8.0[†]	1.8 ± 0.5
4−58	10/10*	8.5 ± 2.4	< 1
4−79	10/10*	5.5 ± 4.8	< 1
4−94	9/10**	7.3 ± 3.0	< 1
6−20	8/10**	8.0	< 1
10−15	9/10**	8.3 ± 0.5	< 1
10−86	9/10**	8.0	< 1
11−34	9/10**	9.8 ± 1.7	< 1
11−36	9/10**	8.5 ± 1.0	< 1
Control (PBS)	10/10*	< 1	< 1

Values that are significantly different from NUF631 FKC value are indicated by asterisks (*, $p < 0.01$; **, $p < 0.05$).

[†] Average agglutination titer (log_2) of sera from five fish against K^- type (NUF44) and K^+ type (NUF631) FKC.

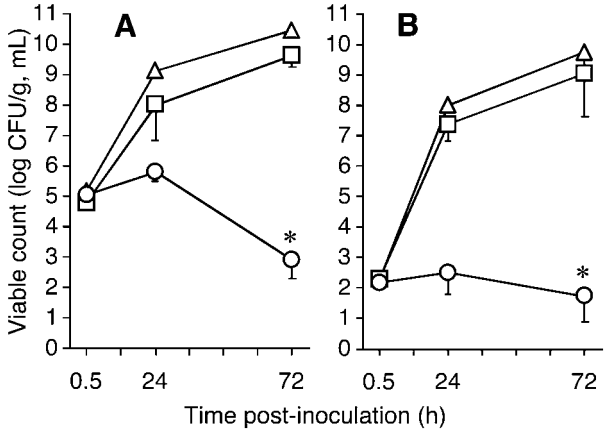

Fig. 1. Changes in viable count of *S. iniae* NUF631 in the kidney (A) and blood (B) of Japanese flounder immunized with FKC. Bacterial suspension (1×10^6 CFU/mL) was intravenously inoculated at 0.1 mL/100 g body weight. Bars represent standard deviations (n = 3). Values that are significantly different ($p < 0.01$) from the value of non-immunized fish are indicated by an asterisk. ○, fish immunized with NUF631 FKC; □, fish immunized with 6-20 FKC; △, non-immunized fish.

Agglutination titers of both NUF631 and mutant immune sera against NUF44 FKC were relatively at a high level (5.5 ± 4.8 to 9.8 ± 1.7). Agglutination titers of NUF631 immune sera against NUF631 FKC were at a low level (1.8 ± 0.5), but those of mutant immune sera against NUF631 FKC were not detected (< 1) (Table 1).

Fate of S. iniae *NUF631 in immunized flounder and plasma*

Viable count of intravenously inoculated NUF631 increased significantly for 72 h in the blood and kidney of flounder immunized with 6−20 FKC as well as non-immunized fish. On the other hand, in fish immunized with NUF631 FKC, viable count of NUF631 increased slightly at 24 h post-inoculation but decreased thereafter (Fig. 1). NUF631 grew in plasma taken from fish immunized with NUF631 FKC and 6-20 FKC as well as in plasma from non-immunized fish at almost the same proliferation rate (data not shown).

Effect of immune sera on phagocytosis and reactive oxygen species (ROS) production of flounder peritoneal macrophages

NUF631 cells opsonized with anti-NUF631 serum were phagocytosed more efficiently than those opsonized with anti-6−20 or normal serum. 6−20 cells opsonized with anti-NUF631, anti-6−20 and normal serum were phagocytosed efficiently at almost the same rate (Fig. 2). Chemiluminescence response of macrophages indicates the production of reactive oxygen species. NUF631 cells opsonized with anti-NUF631 serum strongly promoted ROS production in macrophages compared to those opsonized with anti-6−20 or normal serum. On the contrary, 6−20 cells opsonized with anti-NUF631, anti-6-20 and normal serum promoted the ROS production (Fig. 3).

Transmission electron microscopy of cell surface of S. iniae *pretreated with immune sera*

Electronically dense materials were observed on the capsule of NUF631 cells pretreated with anti-NUF631 serum but not with anti-6−20 or normal serum. They were observed on the cell surface of 6−20 cells pretreated with anti-NUF631 and anti-6−20 serum (Fig. 4).

Detection of serum components bound to bacterial cell surface

Western blot analysis with rabbit antiserum against flounder antibody as a primary antibody revealed a protein band with approximate molecular mass of 70,000 in the anti-NUF631 serum components bound to NUF631 and 6−20 cells, and in the anti-6-20 serum components bound to 6-20 cells (Fig. 5).

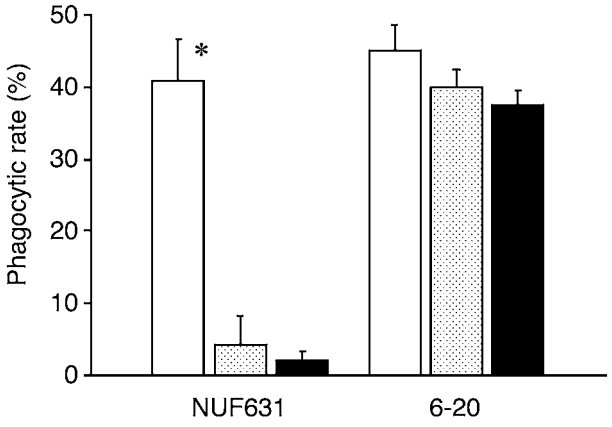

Fig. 2. Phagocytosis of *S. iniae* NUF631 and 6−20 cells by flounder peritoneal macrophages. Values that are significantly different ($p < 0.01$) from the value of bacterial cells opsonized with normal serum are indicated by an asterisk. □, opsonized with anti-NUF631 FKC serum; ▨, opsonized with anti-6−20 FKC serum; ■, opsonized with normal serum.

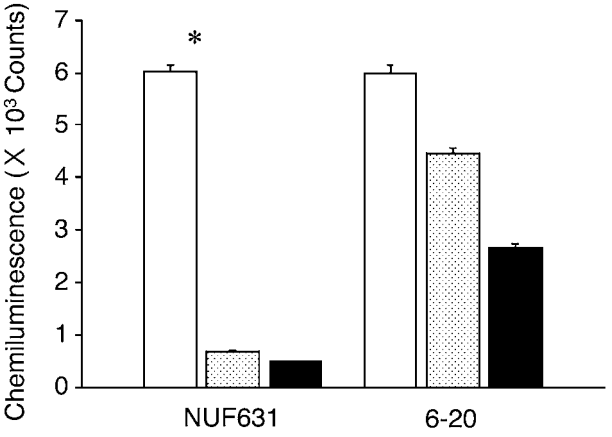

Fig. 3. Chemiluminescence response of flounder peritoneal macrophages to *S. iniae* NUF631 and 6−20 cells. Values that are significantly different ($p < 0.01$) from the value of bacterial cells opsonized with normal serum are indicated by an asterisk. □, opsonized with anti-NUF631 FKC serum; ▨, opsonized with anti-6–20 FKC serum; ■, opsonized with normal serum.

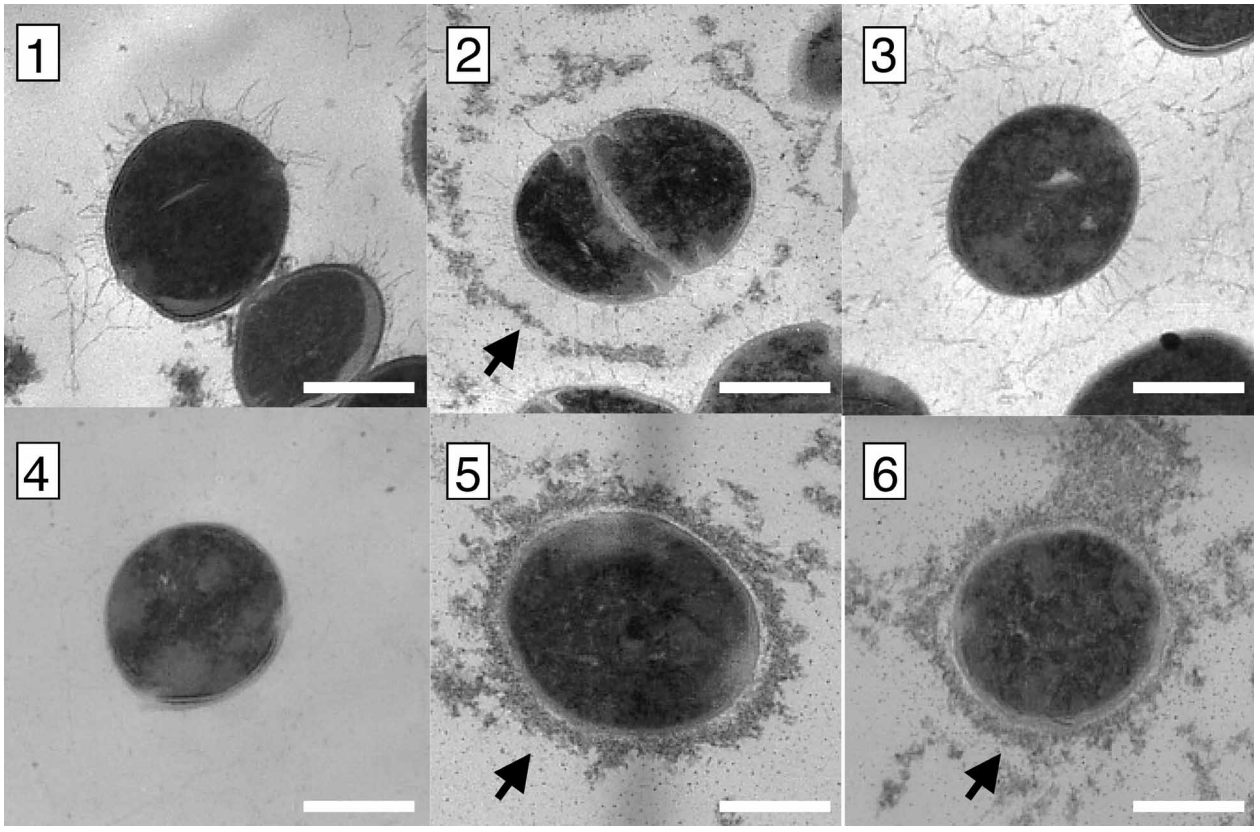

Fig. 4. Transmission electron micrographs of *S. iniae* NUF631 and 6-20 cells pretreated with flounder sera. **1**−**3**, NUF631 cells; **4**−**6**, 6−20 cells. **1** and **4**, pretreated with normal serum; **2** and **5**, pretreated with anti-NUF631 FKC serum; **3** and **6**, pretreated with anti-6−20 FKC serum. Arrow, serum components binding to capsule or cell surface. Bar = 0.5 μm.

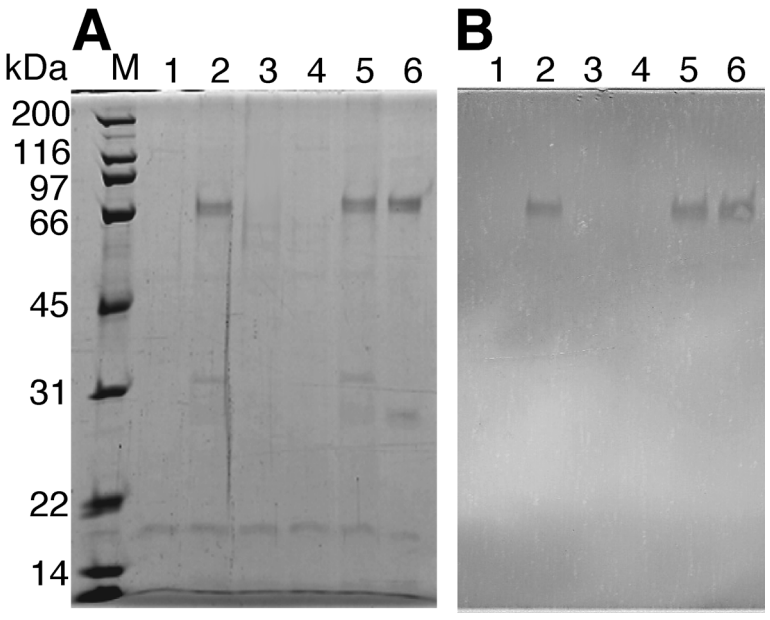

Fig. 5. Detection of flounder serum components bound to the cell surface of *S. iniae* NUF631 and 6−20 by SDS-PAGE (A) and by western blotting with rabbit antiserum against flounder antibody (B). SDS-PAGE gel was stained by Coomassie brilliant blue R. **M**, marker proteins; **1**−**3**, serum components bound to NUF631 cells; **4**−**6**, serum components bound to 6−20 cells. **1** and **4**, normal serum; **2** and **5**, anti-NUF631 FKC serum; **3** and **6**, anti-6−20 FKC serum.

Discussion

The results of immunoprotection test, in which FKC from *S. iniae* NUF631 (capsulated) was effective but those from its isogenic capsule-deleted mutants were ineffective, indicate that the capsular polysaccharides are one of the protective antigens in flounder *S. iniae* infection. Kusuda *et al*. (1996) reported that bacteriostatic activity was observed in the serum from yellowtail *Seriola quinqueradiata* immunized with *Enterococcus seriolicida* FKC and suggested that humoral factors such as antibody and complement played a role in protection against *E. seriolicida* infection as opsonins and antibacterial factors. Sakai *et al*. (1989) described that phagocytic rate of kidney leucocytes but not serum bactericidal activity increased in rainbow trout *Oncorhynchus mykiss* immunized with *S. iniae* FKC. In the present study, viable count of intravenously administrated *S. iniae* NUF631 decreased in the kidney of flounder immunized with NUF631 FKC, whereas viable count of *S. iniae* NUF631 increased in the plasma from flounder immunized with NUF631. So, it is supposed that protection against *S. iniae* infection in flounder immunized with NUF631 FKC is mainly owing to bactericidal activity of phagocytes such as macrophages abundant in the kidney.

The results of phagocytosis experiments using peritoneal macrophages suggest that anti-capsule antibody in the serum or combination of anti-capsule antibody and complement acts as opsonins upon phagocytosis of capsulated *S. iniae* cells and that the participation of anti-capsule antibody is necessary for ROS production by macrophages. Detection of antibody bound to the capsule may serve as an evidence for the implication of the antibody.

The present study demonstrated that *S. iniae* capsule acts as a protective antigen for Japanese flounder and that anti-capsule antibody is an important factor in protective immunity. However, in flounder immunized with NUF631 FKC, not only humoral immunity but also cell-mediated immunity could also be implicated in protection against *S. iniae* infection as indicated by Kakuta *et al*. (2004). Further studies are needed for precise understanding of immunoprotection occurred in fish body.

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