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Serological Characterization of *Streptococcus iniae* Strains Isolated from Cultured Fish in Japan

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ABSTRACT—*Streptococcus iniae* is known as an important marine and freshwater fish pathogen worldwide. In this paper, we characterized two serological phenotypes in Japanese *S. iniae* isolates, mainly from Japanese flounder *Paralichthys olivaceus*. The two phenotypes, designated K^+ and K^-, were distinguished by the presence or absence of capsule. K^- cells agglutinated both with anti-K^- and K^+ type sera, whereas K^+ cells agglutinated only with anti-K^+ type serum. These two types were indistinguishable by any ordinary biological or biochemical characterization tests. Immunodiffusion test demonstrated the common antigens and a K^+ type-specific antigen in the autoclave-extracts of cells. Capsule was observed on the K^+ cells, from which acidic polysaccharides were detected. Strains of K^+ type were virulent for Japanese flounder, whereas those of K^- type were avirulent. There is a direct relationship between the serological phenotypes and the existence of capsule in *S. iniae* with link to its virulence.

Key words: *Streptococcus iniae*, serological phenotype, capsule, virulence, *Paralichthys olivaceus*, Japanese flounder

In 1972, Pier and Madin (1976) examined an Amazon freshwater dolphin *Inia geoffrensis* suffering from golf ball disease and isolated β-hemolytic streptococci from a subcutaneous abscess. They characterized the isolate and named it *Streptococcus iniae* as a new species. Pier *et al*. (1978) described a second isolate of *S. iniae* from *I. geoffrensis*, which differed from the first-described isolate in several biochemical characteristics. These two isolates possessed strain-specific antigens as well as a putative group antigen, which was distinct from any streptococcal group antigens described before (Pier *et al*., 1978). Since then *S.iniae* has been reported to cause serious diseases among various marine and freshwater fish species (Kitao *et al*., 1981; Ogawa *et al*., 1982; Nakatsugawa, 1983; Eldar *et al*., 1994; Perera *et al*., 1994; Sugita, 1996; Bromage *et al*., 1999; Yuasa *et al*., 1999) and recognized as an important fish pathogen in many countries. Comparison of bacteriological characteristics of isolates from these epizootics with those of the isolates from dolphin showed that most fish isolates resembled more to the first-described isolate of *S. iniae* than to the second isolate. The former had been designated as the type strain of this species. It is known that *S. iniae* also causes infectious diseases in humans (Weinstein *et al*., 1996; Lau *et al*., 2003).

Investigations concerning the pathogenesis of *S. iniae* infections have been performed. Yoshida *et al*. (1996b) described the importance of cell capsule in anti-phagocytic potential of the organism. Zlotkin *et al*. (2003) suggested that the abilities of *S. iniae* to survive and multiply within macrophages and to cause their apoptosis play a crucial role in *S. iniae* infection. Neely *et al*. (2002) reported a few putative virulence genes in zebra fish infection model. Implication of phosphoglucomutase (Buchanan *et al*., 2005) and capsule (Miller and Neely, 2005) in the virulence was also suggested. However, information is still insufficient to explain the virulence mechanism of the organism. Vaccination trials with formalin-inactivated *S. iniae* cells were successful in rainbow trout *Oncorhynchus mykiss* (Sakai *et al*., 1987; Eldar *et al*., 1997), yellowtail *Seriola quinqueradiata* (Sako, 1992), tilapia *Oreochromis niloticus* (Klesius *et al*., 2000) and Japanese flounder *Paralichthys olivaceus* (our unpublished results). And now commercially available vaccines are used for preventing *S. iniae* infection in flounder farms. However,

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protective antigens or essential components for vaccines have not been identified yet.

Investigating the serological characteristics of a particular organism is important not only for developing vaccines but also for understanding virulence mechanisms of the organism. However, for *S. iniae* this has scarcely been conducted so far, except that Pier *et al*. (1978) demonstrated group and strain-specific antigens in cell-extracts and that Eldar *et al*. (1997) investigated the antigen recognition by sera of vaccinated fish. Recently Bachrach *et al*. (2001) and Barnes *et al*. (2003) reported the second serotype of *S. iniae* derived from diseased fish in Israel and USA.

To date there are no crucial reports on the serological characteristics of Japanese *S. iniae* isolates. In this paper, we describe the presence and properties of two serological phenotypes in *S. iniae* isolated from diseased fish, mainly from Japanese flounder, in Japan.

Materials and Methods

Bacterial strains

Fifty-four *S. iniae* strains derived from diseased fish are listed in Table 1. Strains with NUF were our original cultures, and the others were kindly supplied by several prefectural and national institutes in Japan. Reference strains, *S. iniae* ATCC29177 and ATCC29178^T, were from the American Type Culture Collection. The strains were routinely cultured in Todd-Hewitt (TH) medium (Difco). Stock cultures were maintained by freezing in liquid nitrogen.

Biological and biochemical characteristics of the strains

Hemolysis was observed on 5% rabbit blood agar. The ability to grow at pH 9.6 and in the presence of bile, and other routine tests were carried out as described by Sakazaki *et al*. (1988). Hyaluronidase and chondroitin sulfatase activities were examined by the plate method described by Smith and Willett (1968). Nicotinamide adenine dinucleotide nucleosidase (NADase) activity and serum opacity reaction were tested by the methods of Lutticken *et al*. (1976) and Hill and Wannamaker (1967), respectively. Deoxyribonuclease (DNase) activity was tested on DNA agar (Nissui). Decarboxylation of amino acids was tested using decarboxylase base Moeller (Difco) supplemented with 1% yeast extract (Difco). Hugh-Leifson medium for oxidation-fermentation (OF) test was also supplemented with 1% yeast extract. Acid production from carbohydrates was tested in BCP semi-solid medium (Eiken).

Rabbit antisera

S. iniae NUF38, NUF44 and NUF631 were cultured in TH broth for 24 h at 30°C. Cells collected by centrifugation were washed twice with 0.01 M phosphate buffered saline (PBS), pH 7.2, suspended at 10 mg (ca. 5×10^9)/mL in PBS containing 0.5% formalin and inactivated for 2 days at room temperature. Formalin-killed cells (FKC) were washed twice with PBS, resuspended at 100 mg/mL in PBS containing 0.02% NaN_3 and stored at 4°C.

One milliliter of the diluted suspension containing 50 mg FKC was emulsified with an equal volume of Freund's complete adjuvant (Wako) and injected subcutaneously to a Japanese white rabbit twice with a 2-wk interval. Two weeks after the second injection the rabbit received an intraperitoneal injection of 50 mg FKC without adjuvant, and 3 wk after the third injection total blood was obtained. Antisera were heated for 30 min at 56°C and stored at –30°C.

In this study anti-NUF38 and anti-NUF44 sera were used as K^- type sera, and anti-NUF631 serum as a K^+ type serum. At the initial stage of this study *S. iniae* NUF38 was used as a representative strain of K^- type, but this strain altered gradually to K^+ type, so the representative strain was changed to NUF44.

Agglutination test

Quantitative agglutination test was performed by serial twofold dilution method with 96-multiwell microplates and PBS as a diluant. FKC was used as the antigen. Agglutinating titer was expressed as a reciprocal of the highest dilution that caused complete agglutination.

Absorption of antiserum

Five hundred microliter of rabbit antiserum was absorbed twice for 2 h at room temperature and overnight at 4°C with 100 mg FKC, and this procedure was repeated until agglutination titer against corresponding FKC became below the detection limit.

Immunodiffusion and immunoelectrophoresis

S. iniae NUF44, NUF631 and ATCC29178^T were cultured in TH broth for 24 h at 27°C. Cell surface antigens were extracted from the bacterial cells by boiling in 0.05 and 0.2 N HCl, autoclaving and treating with 10% trichloroacetic acid (TCA) at 4°C as previously described (Lancefield, 1933; Pier *et al*., 1978). Cell surface antigens of the strains used for virulence test (see below) were also prepared by the autoclave method. Immunodiffusion was performed in 1% agarose (Bio-Rad) in PBS. In immunoelectrophoresis each sample was put in the application well and electrophoresed toward the anode for 90 min at 2 mA/cm in 1 % agarose gel with 0.05 M barbital buffer, pH 8.6. After electrophoresis rabbit antisera were poured into the troughs, and the gels were incubated at room temperature in a humid chamber.

Transmission electron microscopy

Samples for transmission electron microscopy were

Table 1. *Streptococcus iniae* strains used in this study and their agglutinating reactions against rabbit anti-*S. iniae* sera

Strain	Source*[7]	Year	Location	Agglutination titer	
				Anti-NUF38	Anti-NUF631
K^- type					
NUF38*[1]	Japanese flounder	1983	Nagasaki	4096	4096
NUF43	Japanese flounder	1983	Nagasaki	2048	4096
NUF44	Japanese flounder	1983	Nagasaki	1024	4096
NUF45	Japanese flounder	1983	Nagasaki	4096	4096
HS9307*[2]	Japanese flounder	1993	Ehime	4096	2048
FT92019*[3]	Japanese flounder	1992	Tokushima	2048	4096
FT93005*[3]	Japanese flounder	1993	Tokushima	2048	2048
450-3*[4]	Japanese flounder	1993	Ohita	2048	2048
NUF663	Yellowtail	1992	Nagasaki	2048	512
NN9201*[5]	Common mackerel	1992	Nagasaki	1024	4096
K^+ type					
NUF86	Japanese flounder	1984	Nagasaki	< 2	32
NUF630	Japanese flounder	1992	Kumamoto	< 2	16
NUF631	Japanese flounder	1992	Kumamoto	< 2	32
NUF632	Japanese flounder	1992	Nagasaki	< 2	32
NUF633	Japanese flounder	1992	Nagasaki	< 2	16
NUF636	Japanese flounder	1992	Nagasaki	< 2	32
NUF693	Japanese flounder	1993	Nagasaki	< 2	32
NUF694	Japanese flounder	1993	Nagasaki	< 2	32
NUF695	Japanese flounder	1993	Nagasaki	< 2	32
NUF700	Japanese flounder	1993	Ohita	< 2	32
NUF701	Japanese flounder	1993	Ohita	< 2	32
NUF809	Japanese flounder	1997	Nagasaki	ND*[8]	ND
NUF812	Japanese flounder	1997	Ehime	ND	ND
HS9025*[2]	Japanese flounder	1990	Ehime	< 2	32
HS9028*[2]	Japanese flounder	1990	Ehime	< 2	32
HS9104*[2]	Japanese flounder	1991	Ehime	< 2	16
HS9109*[2]	Japanese flounder	1991	Ehime	< 2	32
HS9112*[2]	Japanese flounder	1991	Ehime	< 2	16
HS9301*[2]	Japanese flounder	1993	Ehime	< 2	32
HS9305*[2]	Japanese flounder	1993	Ehime	< 2	32
HS9310*[2]	Japanese flounder	1993	Ehime	< 2	32
HS9311*[2]	Japanese flounder	1993	Ehime	< 2	32
FT91044*[3]	Japanese flounder	1991	Tokushima	< 2	16
FT91048*[3]	Japanese flounder	1991	Tokushima	< 2	32
FT92015*[3]	Japanese flounder	1992	Tokushima	< 2	32
FT93010*[3]	Japanese flounder	1993	Tokushima	< 2	32
464-1*[4]	Japanese flounder	1993	Ohita	< 2	16
464-3*[4]	Japanese flounder	1993	Ohita	< 2	16
466-4*[4]	Japanese flounder	1993	Ohita	< 2	32
487-3*[4]	Japanese flounder	1993	Ohita	< 2	16
496-1*[4]	Japanese flounder	1993	Ohita	< 2	16
496-2*[4]	Japanese flounder	1993	Ohita	< 2	16
NE9207*[5]	Japanese flounder	1992	Nagasaki	< 2	32
F-2*[6]	Japanese flounder	1985	Mie	< 2	32
F-871*[6]	Japanese flounder	1987	Mie	< 2	32
NUF662	Yellowtail	1992	Nagasaki	< 2	64
No.1*[6]	Yellowtail	1985	Mie	< 2	32
No.4*[6]	Yellowtail	1985	Mie	< 2	32
1BL*[6]	Common mackerel	1985	Mie	< 2	64
3K*[6]	Common mackerel	1985	Mie	< 2	64
TNR4*[6]	Rainbow trout	1989	Tochigi	< 2	128
TNR5*[6]	Rainbow trout	1989	Tochigi	< 2	32
PT91036*[3]	Ayu	1991	Tokushima	< 2	32
TT92006*[3]	Threadsail filefish	1992	Tokushima	< 2	32
ATCC29177	Dolphin	1972	USA	SA*[9]	SA
ATCC29178^T	Dolphin	1972	USA	SA	SA

*[1] NUF, Nagasaki University Fisheries; *[2] provided by Ehime Prefectural Fish Disease Control Center; *[3] provided by Fisheries Research Institute, Tokushima Agriculture, Forestry and Fisheries Technology Support Center; *[4] provided by Fisheries Research Institute, Ohita Prefectural Agriculture, Forestry and Fisheries Research Center; *[5] provided by Nagasaki Prefectural Institute of Fisheries; *[6] provided by Dr. Sako, Tohoku National Fisheries Research Institute, Fisheries Research Agency; *[7] Japanese flounder *Paralichthys olivaceus*, yellowtail *Seriola quinqueradiata*, common mackerel *Scomber japonicus*, rainbow trout *Onchorynchus mykiss*, ayu *Plecoglossus altivelis*, threadsail filefish *Stephanolepis cirrhifer*, dolphin *Inia geoffrensis*; *[8] Agglutinating titer was not determined; *[9] Spontaneous agglutination.

prepared as described by Charland *et al*. (1997) with some modifications. Briefly, one loopful of bacterial colonies grown on TH agar was suspended in 2 mL PBS, added with 40 μL of rabbit anti-NUF38 or anti-NUF631 serum and incubated for 3 h at 4°C. Bacterial cells were then fixed with a mixture of 5% glutaraldehyde and 0.15% ruthenium red in 0.1 M cacodylate buffer, pH 7.3, for 2 h at 20°C, embedded in 4% agarose and washed five times with cacodylate buffer containing 0.05% ruthenium red. Postfixation, dehydration and embedding in resin were carried out as described. Thin sections stained with uranyl acetate and lead citrate were examined with a JEM-100S electron microscope (JEOL).

Detection of cellular acidic polysaccharides

Bacterial cells grown in TH broth for 24 h at 27°C were washed and suspended in saline at 0.4 g/mL. Cellular acidic polysaccharides were extracted by autoclaving the suspension for 30 min at 121°C, precipitated from the supernatant by adding four volumes of ethanol and air-dried. Air-dried materials were dissolved in 0.1 M NaCl at a concentration of 0.1%, and 2% cetyltrimethylammonium bromide (CTAB) solution was added slowly while stirring till the amount of CTAB reached double the weight of the air-dried materials. The precipitate formed was discarded, and nine volumes of distilled water were added slowly to the supernatant while stirring. The resultant precipitate composed of crude acidic polysaccharides was collected by centrifugation, dissolved in 1 M NaCl, precipitated again with four volumes of ethanol to remove CTAB and lyophilized.

Electrophoretic detection of acidic polysaccharides was performed by sensitivity-enhanced polyacrylamide gel electrophoresis (SE-PAGE) according to Min and Cowman (1986). Crude acidic polysaccharides were dissolved at 5−10 mg/mL in distilled water, mixed with 1/5 volume of 2 M sucrose dissolved in Tris/borate/EDTA buffer and applied to 10% polyacrylamide gel, 8.5 cm in length. Electrophoresis was conducted at a constant current of 25 mA for 6 h. Acidic polysaccharides in the gel were fixed with alcian blue and visualized by silver-stain (Merril *et al*., 1981).

Virulence test

Four strains of both K^- type (NUF44, HS9307, FT93005 and 450-3) and K^+ type (NUF631, HS9301, FT92015 and 466-4) were cultured on TH agar for 18 h at 27°C. Bacterial cells were suspended in PBS, and tenfold serial dilutions were prepared and injected intramuscularly to juvenile Japanese flounder (50.0 ± 11.2 g body weight), five fish per dilution. Inoculation dosages were 10^2, 10^4, 10^6 and 10^8 CFU/100 g body weight. Injected fish of each strain and dilution were held in a 30-L circular polycarbonate aquarium with constant water flow and aeration and reared for 14 days without feeding. Fish died were autopsied, and the cause of deaths was examined by isolating bacteria from the kidneys on TH agar and testing agglutinability of isolated bacteria with anti-NUF631 serum. Water temperature during the experimental period ranged from 25 to 28°C.

Immuno-protection test

Two groups of ten juvenile flounder, average body weight of 44.5 g, were vaccinated intraperitoneally with FKC (2 mg/mL) of *S. iniae* NUF44 or NUF631 at 0.5 mL/100g body weight. As a control, ten fish received PBS. The fish were challenged by intraperitoneal inoculation with a fresh culture of NUF631 at a dose of 5.7×10^5 CFU/100g body weight 4 wk after vaccination. Average water temperature during the vaccination period and after challenge was 24.6 and 24.7°C, respectively.

Results

Agglutinating reactions of the strains

Fifty-four *S. iniae* strains isolated from fish were serologically divided into two groups, designated K^+ type and K^- type, by agglutinating reactions against two kinds of antiserum used in this study (Table 1). Strains of K^- type agglutinated with anti-NUF38 and anti-NUF631 sera, while strains of K^+ type agglutinated with anti-NUF631 serum but not with anti-NUF38 serum. Agglutination titers of strains of K^+ type against anti-NUF631 serum (16 to 128) were lower than those of K^- type (512 to 4096). FKC of two ATCC strains showed

Table 2. Agglutinating reactions of two representative strains of *Streptococcus iniae* against absorbed and unabsorbed antisera

Strain	Agglutination titer					
	Anti-NUF44			Anti-NUF631		
		Absorbed with			Absorbed with	
	Unabsorbed	NUF44	NUF631	Unabsorbed	NUF44	NUF631
NUF44 (K^- type)	4096	< 4	4096	8192	< 4	4096
NUF631(K^+ type)	< 4	ND	ND	128	128	< 4

ND, not determined.

spontaneous agglutination, but their autoclaved cells reacted with both antisera, indicating that they belonged to K^- type (data not shown).

To clarify the serological relationship between the types, agglutination of representative strains against absorbed antisera was examined. As a result NUF44 (K^- type) agglutinated with anti-NUF38 serum absorbed with NUF631 and anti-NUF631 serum absorbed with NUF631, while NUF631 (K^+ type) agglutinated with anti-NUF631 serum absorbed with NUF44 (Table 2). These

Table 3. Presence of antigens A, B and C in various cell extracts from *S. iniae* ATCC29178^T and NUF44 (K^- type) and NUF631 (K^+ type) demonstrated by immunoprecipitating reactions in agar

Strain	Antigens present in the extracts			
	Autoclave	0.05 N HCl	0.2 N HCl	10% TCA
ATCC29178	A C	A	A	
NUF44	A C	A	A	
NUF631	ABC	AB	A	B

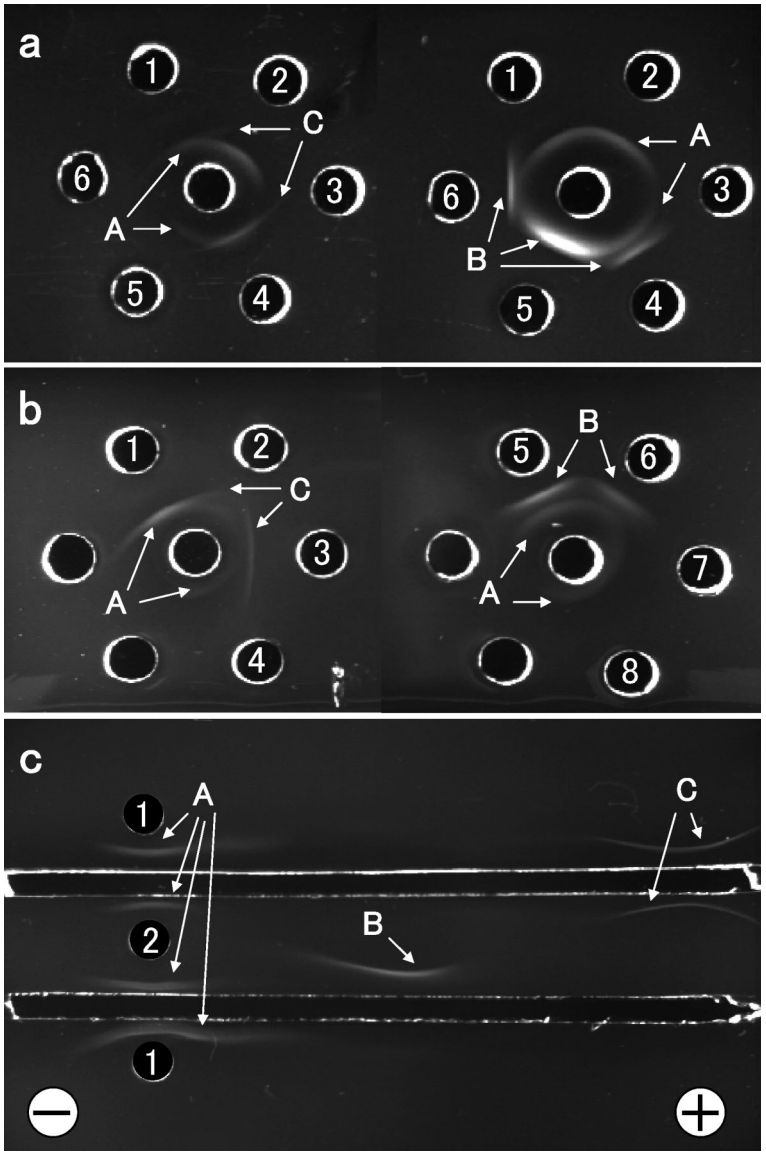

Fig. 1. Immunoprecipitating reactions of cell surface antigens of *Streptococcus iniae* NUF44 (K^- type) and NUF631 (K^+ type). **a**: left center well, anti-NUF44 serum; right center well, anti-NUF631 serum; 1, autoclave-extracts of NUF44 cells; 2, 0.05 N HCl-extracts of NUF44 cells; 3, 10% TCA-extracts of NUF44 cells; 4, autoclave-extracts of NUF631 cells; 5, 0.05 N HCl-extracts of NUF631 cells; 6, 10% TCA-extracts of NUF631 cells. **b**: left center well, anti-NUF44 serum; right center well, anti-NUF631 serum; 1, autoclave-extracts of NUF44 cells; 2, 10% TCA-treated autoclave-extracts of NUF44 cells; 3, 0.2 N HCl-treated autoclave-extracts of NUF44 cells; 4, 0.2 N HCl-extracts of NUF44 cells; 5, autoclave-extracts of NUF631 cells; 6, 10% TCA-treated autoclave-extracts of NUF631 cells; 7, 0.2 N HCl-treated autoclave-extracts of NUF631 cells; 8, 0.2 N HCl-extracts of NUF631 cells. **c**: upper trough, anti-NUF44 serum; lower trough, anti-NUF631 serum; 1, autoclave-extracts of NUF44 cells; 2, autoclave-extracts of NUF631 cells. A, antigen A; B, antigen B; C, antigen C.

results suggest both types possess common antigen(s) and that K^+ type possesses type-specific antigen(s), which interrupts the agglutinating reaction between the common antigen and the corresponding antibody.

Transformation of the serological types

Serial subcultures (more than 30 times) of strains belonging to K^+ type on yeast extract agar (1% polypepton, 0.5% yeast extract, 0.5% NaCl, 1.5% agar and pH 7.2) or TH agar did not cause transformation of these strains to K^- type, but such transformation occurred after two or three subcultures on TH agar containing 0.015% 2, 3, 5-triphenyltetrazolium chloride (TTC). Chemical treatments changed agglutinating properties of bacterial cells against the antisera, that is, bacterial cells of K^+ type became agglutinable to anti-NUF38 serum after boiling for 15 min in saline containing at least 0.025 N HCl or 1.25% TCA. Autoclaving for 90 min at 121°C, treatment with 8% sodium dodecylsulfate for 60 min at 37°C or digestion with 4% trypsin overnight at 37°C did not change their agglutinating properties (data not shown).

Biological and biochemical characteristics of the strains

The strains of both K^- type and K^+ type appeared to have identical biological and biochemical characteristics as *S. iniae* ATCC29178^T. The two types were indistinguishable by any tests carried out in this study (data not shown).

Analyses of cell-surface antigens by immunodiffusion and immunoelectrophoresis

Three kinds of major antigen, designated antigen A, B and C, were detected in the cell extracts from *S. iniae*

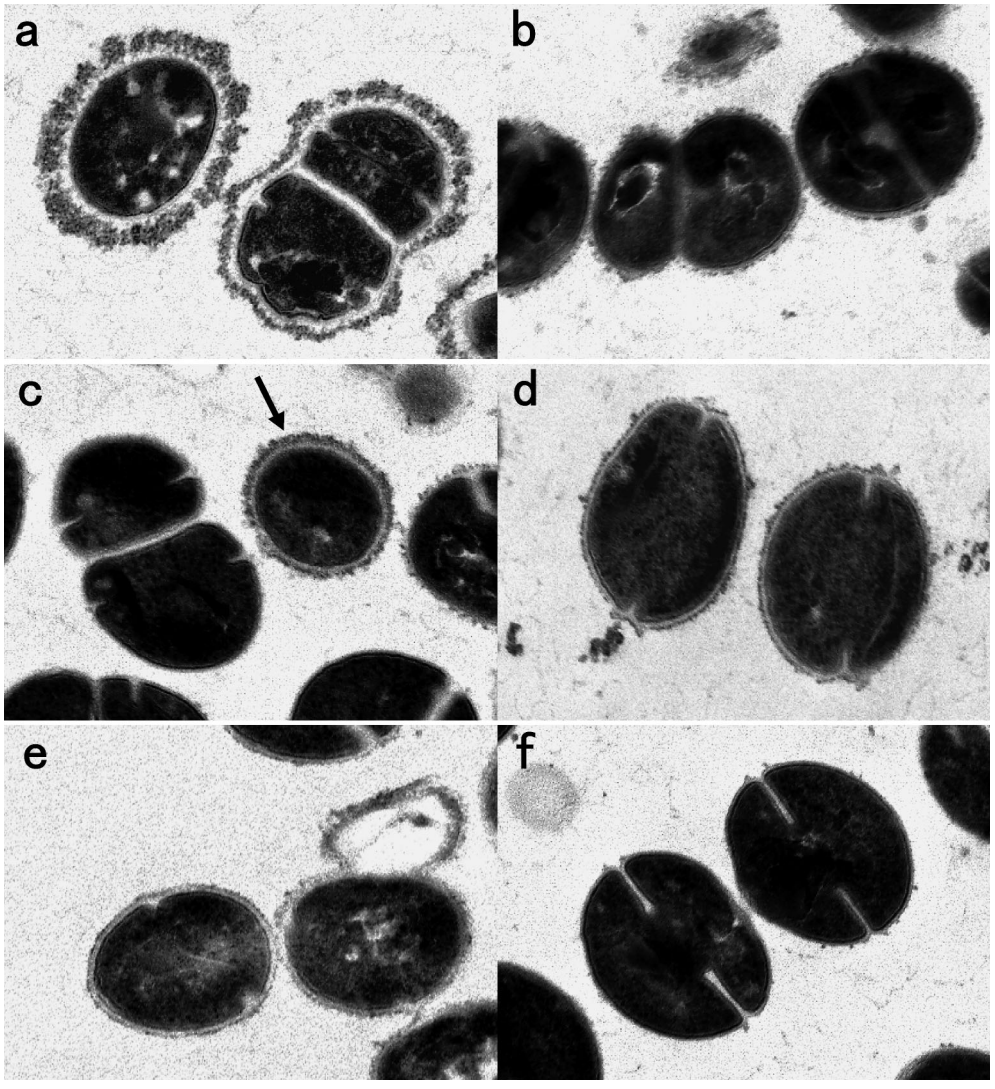

Fig. 2. Transmission electron micrographs of K^+ and K^- cells of *Streptococcus iniae*. **a** and **d**, NUF631 (K^+ type) cells; **b**, **e** and **f**, NUF44 (K^- type) cells; **c**, 450-3 (K^- type) cells. **a**, **b** and **c**, pretreated with anti-NUF631 serum; **d** and **e**, pretreated with anti-NUF38 (K^- type) serum; **f**, non-pretreated. **Note**: (a) Capsules were observed on the cells of NUF631 pretreated with anti-NUF631 serum; (c) a few slightly encapsulated cells (arrow) were observed among a lot of non-capsulated cells of 450-3.

ATCC29178^T and NUF44 (both K^- type) and NUF631 (K^+ type) by immunodiffusion and immunoelectrophoresis using anti-NUF44 and anti-NUF631 sera (Table 3). Two (antigens A and C) of them were common among the three strains and one (antigen B) was NUF631-specific. Antigen A was extracted by autoclaving and heating in HCl and reacted both with anti-NUF44 and anti-NUF631 sera. Antigen B was extracted by autoclaving, boiling in 0.05 N HCl and incubating in 10% TCA at 4°C. It was also extractable by boiling without HCl. Antigen C was extracted by autoclaving and reacted only with anti-NUF44 serum (Fig. 1a). This antigen was suspected to be a protein or glycoprotein since treatment of autoclave-extracts with 10% TCA resulted in the disappearance of the precipitation line, whereas antigen A might be a carbohydrate, because the precipitation line was still formed after such treatment (Fig. 1b). Antigen A was acid-stable, whereas antigen B might be partially acid-stable, because treatment of autoclave-extracts by heating in 0.2 N HCl resulted in the disappearance of the precipitation line (Fig. 1b) and the precipitation line of 0.05 N HCl-extracts was weaker than those of autoclave- and TCA-extracts (Fig. 1a). In immunoelectrophoresis antigen A formed a precipitating line just below or above the application well, indicating that it is electrically neutral. In contrast, antigens B and C formed the lines at the anode side of the application wells, indicating that they are negatively charged (Fig. 1c).

In immunodiffusion of the strains used in the virulence test the strains of K^+ type formed two precipitation lines with anti-NUF631 serum due to antigens A and B as NUF631 did, whereas those of K^- type formed one precipitation line due to antigen A except one strain, 450-3, which formed a faint line due to antigen B besides antigen A (data not shown).

Electron microscopic observation of bacterial cells

Cells of *S. iniae* NUF631 (K^+ type) pretreated with anti-NUF631 serum exhibited a capsular layer around them (Fig. 2a). Such a layer was also observed around the cells of other strains belonging to K^+ type (data not shown), but not observed on the cell surface of NUF44 (Fig. 2b) or other strains of K^- type except for 450-3, in which a similar but much thinner layer was observed on a few cells (Fig. 2c). The cell surfaces of NUF44 and NUF631 pretreated with anti-NUF38 serum (Figs. 2d and 2e) was deeply stained comparing with those not pretreated (Fig. 2f), but the appearance was distinct from the capsule that observed on the surface of K^+ cells pretreated with anti-NUF631 serum.

SE-PAGE of cellular acidic polysacchalides

Smear-like staining of all samples derived from K^+ type was observed in the SE-PAGE gel, while no obvious staining was observed in any samples from K^- type (Fig. 3). The front end of the smear exhibited a typical ladder-like banding pattern.

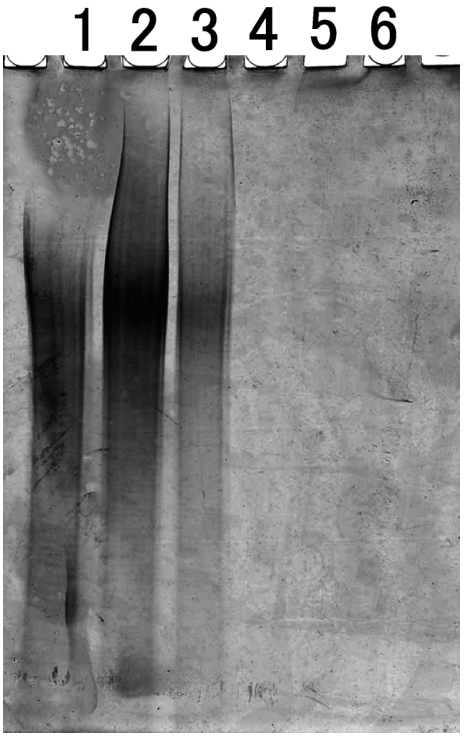

Fig. 3. Sensitivity-enhanced polyacrylamide gel electrophoresis (SE-PAGE) of acidic polysaccharide fraction of *Streptococcus iniae* NUF631 (1), NUF809 (2), NUF812 (3), NUF44 (4), FT93005 (5) and 450-3 (6). The former three are K^+ type and the latter three are K^- type. Acidic polysaccharides were detected by alcian blue-silver stain.

Virulence

Virulence tests revealed that all four strains of K^+ type were virulent ($LD_{50,} < 10^{3.4}$), while all four strains of K^- type were avirulent ($LD_{50,} > 10^{8.0}$) (Table 4). *S. iniae* was isolated from all dead fish. None of the fish inoculated with K^- strains died or exhibited any signs of disease.

Table 4. Virulence (LD_{50}) of selected K^+ and K^- strains of *Streptococcus iniae* in Japanese flounder

Strain	LD_{50}*
K^+ type	
NUF631	$< 10^{2.3}$
HS9301	$< 10^{2.4}$
FT92015	$< 10^{3.0}$
466-4	$< 10^{3.4}$
K^- type	
NUF44	$> 10^{8.0}$
HS9307	$> 10^{8.0}$
FT93005	$> 10^{8.1}$
450-3	$> 10^{8.1}$

* CFU/100 g body weight

Immuno-protection

All control and NUF44 FKC-vaccinated fish died within 7 days. On the other hand 80% of NUF631 FKC-vaccinated fish survived at 2 wk post-challenge (Fig. 4). *S. iniae* was isolated from all dead fish.

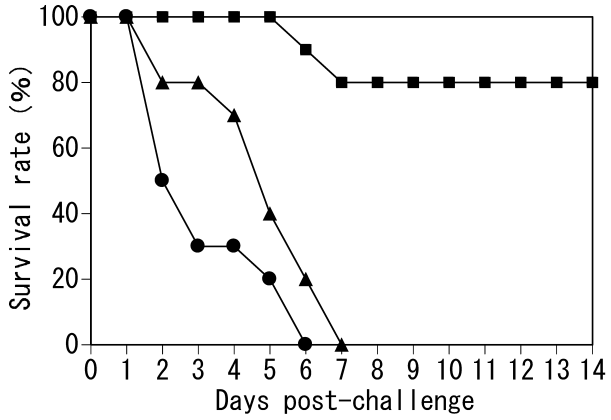

Fig. 4. Immuno-protection in Japanese flounder (n = 10) vaccinated with formalin-killed cells (FKC) of *Streptococcus iniae* NUF631 (K^+ type) and NUF44 (K^- type) against intraperitoneal inoculation of live cells of NUF631. ■, NUF631 FKC; ●, NUF44 FKC; ▲, control.

Discussion

In this paper, we described the existence of two serological types, i.e. K^+ type and K^- type, among strains of *S. iniae* isolated from diseased fish in Japan. However, from the cross-absorption test it was suggested that both types had common surface antigens and that K^+ type had a specific cell surface structure that might prevent the interaction between common antigens and their corresponding antibodies. Such relation between two types resembles to that of *Lactococcus garvieae*, a pathogen of fish lactococcicosis, in which two serological types were designated KG+ and KG- (Kitao, 1982). Strains of KG+ type agglutinated both with anti-KG+ and anti-KG- sera while those of KG- type agglutinated only with anti-KG- serum. It was also reported that the transformation of KG- strains to KG+ type occurred as a result of successive subcultures on artificial agar media or a few subcultures on agar media containing TTC (Kitao, 1982; Alim *et al*., 1996). In this study, the transformation in *S. iniae* strains did not occur by subculturing at least 30 times on TH agar, but occurred after subculturing two or three times on TH agar containing TTC. Physicochemical treatments such as autoclaving, boiling in 0.2 N HCl, incubating with deoxycholate, heating in 10% TCA and digesting with trypsin did not cause the transformation of KG- *L. garvieae* cells to KG+ type (Kitao, 1982), while in *S. iniae* boiling in HCl or TCA caused the transformation, probably due to changing of the surface structure.

There are several reports concerning the diversity of *S. iniae* strains in biochemical characteristics (Pier *et al*., 1978; Sakai *et al*., 1993; Stoffregen *et al*., 1996; Dodson *et al*., 1999), genomic structure (Eldar *et al*., 1997; Fuller *et al*., 2001) and serological properties (Pier *et al*., 1978; Klesius *et al*., 2000; Bachrach *et al*., 2001; Barnes *et al*., 2003). In the present study, though genomics were not investigated, the two serological types were indistinguishable by any biochemical tests performed.

Immunodiffusion and immunoelectrophoresis of autoclave-extracts of *S. iniae* cells demonstrated two common antigens (antigens A and C) and a K^+ type-specific antigen (antigen B). Antigens A and B are supposed to be carbohydrates, because they are stable upon TCA treatment, while antigen C may be a protein or glycoprotein as the precipitation line disappeared after TCA treatment. Antigen B is less stable in acid than antigen A and thought to be an acidic polysaccharide from electric mobility. Antigen A is probably the group antigen of *S. iniae*, because it is acid-extractable and has carbohydrate nature (Pier *et al*., 1978; Bachrach *et al*., 2001).

It is known that specific antibodies stabilize bacterial capsules (Bayer and Thurow, 1977; Charland *et al*., 1997; Yoshida *et al*., 1997; Vanrobaeys *et al*., 1999). Electron microscopic examination demonstrated a capsule on the K^+ cells pretreated with the K^+ serum, whereas capsular structure was not observed on the K^- cells pretreated either with K^+ or K^- serum. This phenomenon for capsulation also resemble to the observation on KG- and KG+ cells in *L. garvieae* (Yoshida *et al*., 1997). Thus, K^+ and K^- type could be categorized to capsulated and noncapsulated phenotypes, respectively. The capsule may be a structure that inhibits cell surface common antigen-mediated agglutination of K^+ cells. It was also found that some strains of K^- type exhibited slightly encapsulated cells such as 450-3 (Fig. 2c).

In general acidic polysaccharide shows a characteristic ladder-like banding pattern in polyacrylamide gel electrophoresis, each band corresponds to a molecule consisted of repeated oligosaccharide units (Min and Cowman, 1986; Pelkonen *et al*., 1988). Smear and ladder-like banding pattern was observed in SE-PAGE of autoclave-extracts from K^+ cells, but not from K^- cells, suggesting K^+ type possesses an acidic polysaccharide on the cell surface.

In this study, all the strains of K^+ type tested were virulent, whereas those of K^- type were avirulent. This is also similar to the results of virulence tests of *L. garvieae* (Alim *et al*., 1996; Yoshida *et al*., 1996a; Barnes *et al*, 2002). In *L. garvieae* it was suggested that the resistance to opsonophagocytosis and complement-mediated killing contributed to the virulence of KG- strains (Yoshida *et al*., 1997; Barnes *et al*., 2002). There are many reports that described the involvement

of capsules in the pathogenicity of bacteria (Wessels and Bronze, 1987; Smith *et al*., 1999; Magee and Yother, 2001). The capsulated phenotypes of *L. garvieae* and *S. iniae* were virulent, so their capsules are supposed to function as a virulence factor.

All the strains belonging to K^+ type or K^- type used in this study were derived from diseased fish. The reason why the avirulent phenotype (K^- type) was isolated from diseased fish is unknown. However, it is often experienced that bacterial colonies of K^- type were cultivated from survivors in challenge experiments using strains of K^+ type (our unpublished observation). Thus, transformation from K^+ type to K^- type may occur in fish. So, there raises the possibility that the strains of K^- type were isolated from fish recovering from streptococcosis or suffering from a complicated disease.

Although only one strain from each serological type was used in immuno-protection test, it is possible to speculate that the capsular components of *S. iniae* play a role in the protective immunity, because capsular polysaccharides are thought to be the major immunogenic components in vaccines such as pneumococcal, staphylococcal, meningococcal and group B streptococcal vaccines (Artz *et al*., 2003; O'Riordan and Lee, 2004; Lindahl *et al*., 2005; Harrison, 2006).

In summary, Japanese *S. iniae* isolates, mainly from Japanese flounder, were divided into two serological phenotypes, one of which exhibited a type-specific anionic polysaccharide antigen and capsule and is virulent. In future studies it is important to prove the relation between the type-specific antigen and capsule and to clarify their roles in the pathogenicity of this organism. Investigations on genetic mechanisms of transformation from K^+ to K^- type or from K^- to K^+ type are also needed for understanding the occurrence and relation of two types.

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