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Virulence Attenuation of Capsular Polysaccharide-deleted Mutants of *Streptococcus iniae* in Japanese Flounder *Paralichthys olivaceus*

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ABSTRACT—*Streptococcus iniae* is known as an important bacterial pathogen causing serious damage in fish culture industry worldwide. Although the capsule of this pathogen is suggested to play a role in the disease process, it has not been verified yet. In this study we created isogenic uncapsulated mutants of *S. iniae* NUF631 by Tn*916* transposon mutagenesis and investigated the role of capsule in the virulence and resistance to phagocytic activities of peritoneal macrophages of Japanese flounder *Paralichthys olivaceus*. Mutants transformed from K^+ phenotype to K^- phenotype were all uncapsulated. They exhibited neither K^+ type-specific precipitation line in an immunodiffusion test nor ladder-like banding pattern characteristic for bacterial acidic polysaccharides in polyacrylamide gel electrophoresis. Their virulence to Japanese flounder was highly attenuated. In contrast to the mutant strains, the parent capsulated strain resisted phagocytic and bactericidal activities of the macrophages and prevented chemiluminescence reaction of the macrophages upon phagocytosis. From these results, it is verified that the *S. iniae* capsule is an important factor for intramacrophage survival of the pathogen.

Key words: *Streptococcus iniae*, capsule, transposon mutagenesis, virulence, *Paralichthys olivaceus*, Tn*916*, macrophage, chemiluminescence

Streptococcus iniae is an important pathogen causing systemic infection in fish. *S. iniae* infection has been reported worldwide in many intensively cultured fish species (Kitao *et al*., 1981; Nakatsugawa, 1983; Perera *et al*., 1994; Elder *et al*., 1999; Bromage *et al*., 1999; Yuasa *et al*., 1999). In Japanese flounder *Paralichthys olivaceus*, *S. iniae* causes acute septicemic disease during the warm summer months.

So far, there are several reports dealing with virulence mechanisms of *S. iniae* in fish streptococcosis (Neely *et al*., 2002; Zlotkin *et al*., 2003; Buchanan *et al*., 2005), and some are on the importance of capsule (Yoshida *et al*., 1996; Miller and Neely, 2005). Kanai *et al*. (2006) reported the presence and properties of two serological phenotypes, designated K^+ type and K^- type, in Japanese *S. iniae* isolates, mainly from Japanese flounder. They showed that K^+ type exhibited a type-specific antigen and acidic polysaccharides in the autoclave-extracts of cells and a capsule on the cell surface, and that strains of K^+ type were virulent for Japanese flounder whereas those of K^- type were avirulent. Thus, it is supposed that the capsule plays a role in pathogenesis of streptococcosis in Japanese flounder and that the capsule is constructed with the acidic polysaccharides, which are the K^+ type-specific antigen.

In this study, we created isogenic mutant strains that were changed from K^+ type to K^- type by transposon mutagenesis. We investigated the relationship between the capsule, acidic polysaccharides and K^+ type-specific antigen, and the importance of these items in *S. iniae* virulence through examining the resistance to phagocytic and bactericidal activities of flounder peritoneal macrophages.

Materials and Methods

Bacteria

S. iniae NUF631 was used as a representative strain of the K^+ phenotype (Kanai *et al*., 2006). *S. iniae* NUF631-SR, a streptomycin resistant strain (MIC is ≧1,600 μg/mL) derived from NUF631 by several subcul-

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tures on Todd-Hewitt agar (TH; Difco Laboratories) supplemented with increasing amount of streptomycin, was used as the recipient strain for transposon mutagenesis. *Enterococcus faecalis* CG110, which harbored Tn*916*, an 18-kb transposon encoding a tetracycline resistance gene (Gawron-Burke and Clewell, 1982; Caparon and Scott, 1989), was used as the donor strain.

Transposon mutagenesis and isolation of K^+ type-specific antigen-deleted mutants

E. faecalis CG110 and *S. iniae* NUF631-SR were grown for 18 h at 27°C in TH broth. Both cultures were mixed at a ratio of 1:5 and passed through a membrane filter (pore size is 0.45 μm), and the filter was placed on TH agar supplemented with 5% horse blood and incubated for 18 h at 27°C. Cultured bacterial cells on the filter were scraped off, suspended in TH broth and plated on TH agar supplemented with streptomycin (1,600 μg/mL; to inhibit *E. faecalis* growth) and tetracycline (50 μg/mL; to select *S. iniae* Tn916 transconjugants) (Shimoji *et al*., 1994). Each colony grown was cultivated for 24 h at 27°C in TH broth and mixed with rabbit anti-NUF44 serum for bacterial agglutination test. Transconjugants changed from non-agglutinable to agglutinable with anti-NUF44 serum, i.e., changed from K^+ to K^- phenotype, (Kanai *et al*., 2006) were stored.

Preparation of digoxigenin-labelled Tn916 probe and Southern hybridization

Tn*916* possesses one *Hin*dIII restriction site in its sequence. An oligonucleotide, Tn-1 (5'-GAGGTCATT-CTTAGTGGAGAAATCCCTGCTCGGTG T-3'), which is a part of the tetracycline gene sequence located in one Tn*916 Hin*dIII fragment, was synthesized and end-labeled with digoxigenin (DIG) using DIG Oligonucleotide 3'-End Labeling Kit (Roche Applied Science). *S. iniae* chromosomal DNAs were prepared using AquaPure Genomic DNA Isolation Kit (BIO-RAD). The DNAs from the parent (NUF631) and mutant strains were digested with *Hin*dIII and transferred to nylon membrane (Hybond-N^+; GE Healthcare Bio-Sciences). Southern hybridization was performed according to the DIG systems user's guide (Roche Applied Science).

Determination of nucleotide sequences at the Tn916-inserted sites

Chromosomal DNAs of mutant strains were digested with *Hin*dIII, and the resultant fragments were self-ligated using DNA Ligation Kit Ver.2.1 (TaKaRa Bio). Inverse PCR was performed with primers, Tn-10 (5'-CTATCCTACAG CGACAGCCAGTGAACTTTC-3') and Tn-12 (5'-GCTGGCAGGAATACTTACT TGAATCA-TGCG-3'), which were oppositely directed primers designed from the both end regions of one Tn*916 Hin*dIII fragment. Amplification was performed for 30 cycles, with each cycle consisting of 60 s of denaturing at 94°C, 45 s of annealing at 50°C and 9 min of extension at 72°C with MyCycler thermal cycler (BIO-RAD). PCR products were cloned using pGEM-T Easy Vector Systems (Promega). Nucleotide sequences were determined with BigDye Terminator Ver. 3.1 Cycle Sequencing Kit and ABI 3100 DNA sequencer (Applied Biosystems).

The nucleotide sequence data were analyzed using DNASIS program (Hitachi Software) and compared with known sequences in public databases using the National Center for Biotechnology information (NCBI) BLASTX software available at www.ncbi.nlm.nih.gov.

Virulence test

S. iniae NUF631 and mutant strains were cultured and suspended at 10^{10} CFU/mL in phosphate-buffered saline (PBS). Five Japanese flounder (103.8 ± 12.6 g) were intramuscularly inoculated with bacterial suspension at 0.1 mL/100 g body weight. Number of death was monitored daily for 10 days post-inoculation. Reisolation of bacteria from dead and survived fish was carried out from the kidney and brain, and slide agglutination test with rabbit anti-NUF631 serum was conducted to identify the isolates.

Fate of S. iniae *in Japanese flounder*

S. iniae NUF631 and mutant strains were cultured and suspended at 10^6 CFU/mL PBS. Twelve Japanese flounder (183.9 ± 23.5 g) were intravenously inoculated with bacterial suspension at 0.1 mL/100 g body weight. Three flounder were taken up at 30 min and 24 h-intervals post-inoculation, and the blood and kidney were sampled. Viable cell numbers of *S. iniae* in the samples were calculated by colony count method.

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

Blood was collected from flounder with a heparinized syringe, and the plasma was separated by centrifugation at 3,000 rpm for 15 min and kept on ice until use. *S. iniae* NUF631 or mutant cell suspensions were adjusted to 10^4 CFU/mL PBS. Bacterial suspension and the plasma were mixed at a ratio of 1:10 and incubated for 24 h at 27°C and 10% CO_2. Changes in viable count of *S. iniae* in the mixture were measured by colony count method.

Bacterial cell surface hydrophobicity

Bacterial cell surface hydrophobicity was determined by the method of Rosenberg *et al*. (1980). Bacterial cells were harvested, washed twice and suspended in PUM buffer ($K_2HPO_4 \cdot 3H_2O$ 22. g, KH_2PO_4 7.26 g, urea 1.8 g, $MgSO_4 \cdot 7H_2O$ 0.2 g in distilled water to 1,000 mL, pH 7.1) at an optical density of 0.6 at 660 nm, and 1.16 mL of the bacterial suspension was layered on 0.04 mL of n-hexadecane in a glass tube. After incubation for 20 min at 27°C, the tube was agitated vig-

orously for 2 min and allowed to stand for 15 min for separation. The percentage of bacteria partitioned into the hydrocarbon phase was calculated by the following formula: 100 × (A660 of initial bacterial suspension − A660 of hydrocarbon-treated aqueous phase)/ A660 of initial bacterial suspension, where A660 = absorbance at 660 nm.

Preparation of flounder peritoneal macrophages

Blood was collected from the caudal vein of 1-year-old Japanese flounder, and the serum was separated after clotting for 2 h at room temperature and centrifuging at 3,000 rpm for 15 min and kept on ice until use for opsonizing bacteria. Then 10 mL of Dulbecco's PBS supplemented with 0.5% NaCl (DPBS) and heparin (40 unit/mL) was injected into the peritoneal cavity and, after gently massaging the abdomen, the injected DPBS containing the peritoneal cells was withdrawn with a syringe. The peritoneal cells were suspended in 0.1 mL of DPBS and separated by Percoll continuous density gradient. Peritoneal macrophage fraction was collected and washed with DPBS. Cell viability was usually more than 99% as determined by trypan blue exclusion test. The cell density was then adjusted to 2×10^7 cells/mL in RPMI 1640 medium (Nissui Pharmaceutical) supplemented with 10% fetal bovine serum and 0.5% NaCl (RPMI medium). Macrophage suspension was incubated for 1 h at 27°C in an atmosphere of 10% CO_2 prior to the following experiments. Centrifuge tubes and glass wares pretreated with silicone were used throughout the experiment.

Phagocytosis assay

Equal volumes of bacterial suspension (1×10^9 CFU/mL) and fresh normal flounder serum were mixed and incubated for 20 min at 27°C. Macrophage suspension and opsonized bacteria were mixed at a multiplicity of infection (MOI) of 1:25 and incubated for 30 min at 27°C and 10% CO_2. Then smears of the mixture were prepared, stained by May-Giemsa and examined by light microscopy. The assay was repeated by three independent experiments. Phagocytic rate was calculated as (no. of macrophage with engulfed bacteria / 200 macrophages) × 100 (Charland *et al*., 1996).

Intracellular killing assay

Bacteria (2×10^8 CFU/mL) were opsonized as above. Macrophage (1×10^7 cells/mL) and opsonized bacteria were mixed at MOI of 1:100 and incubated for 30 min at 27°C and 10% CO_2. To kill extracellular bacteria, gentamicin (1 mg/mL) was added to the mixture at a final concentration of 100 μg/mL and incubated for 1 h at 27°C and 10% CO_2. Then extracellular bacteria were removed by centrifugation for 10 min at 20°C and 1,000 rpm, and the macrophages were washed three times with DPBS and resuspended in fresh RPMI medium. At 1 h-intervals, 0.1 mL of the cell suspension was taken and added with nine volumes of sterile distilled water. After standing for 2 min at 4°C and vortexed, cell suspension was serially diluted in PBS and plated on TH agar to determine the number of viable intracellular bacteria. The assay was repeated by three independent experiments. Intracellular killing was expressed as the ratio to the initial intracellular viable count.

Chemiluminescence assay

One hundred microliter of macrophage suspension (2×10^7 cells/mL HBSS) and the same volume of 40 μM MCLA (2-methyl-p-methoxyphenyl-3, 7 dihydroimidazo [1, 2-a] pyradin 3-one) in HBSS were mixed in each well of OptiPlate-96F (Perkin Elmer). The plate was placed in Luminescencer JNR II (ATTO) for 10 min at room temperature. Finally, 20 μL of bacteria (6×10^9 CFU/mL HBSS) opsonized with flounder normal serum was added to each well, and the chemiluminescence intensity was measured for 25 min.

Miscellaneous methods

Preparation of rabbit antisera against *S. iniae* NUF44 (K^- phenotype) and NUF631, immunodiffusion test in agar, detection of acidic polysaccharides by sensitivity-enhanced polyacrylamide gel electrophoresis (SE-PAGE) and assessment of the capsule by transmission electron microscopy were carried out as described previously (Kanai *et al*., 2006).

Statistics

Significant differences were analyzed by using ANOVA.

Results

Isolation and characterization of K^+ type-specific antigen deleted mutants

Filter mating method was used to transfer Tn*916* from *E. faecalis* CG110 to *S. iniae* NUF631-SR. Approximately 3,000 transconjugants were isolated from TH agar containing streptomycin and tetracycline, and among them eleven transconjugants have changed from non-agglutinable to agglutinable with anti-NUF44 serum, indicating that they transformed from K^+ type to K^- type. None of them demonstrated a K^+ type-specific precipitation line in immunodiffusion test, ladder-like banding pattern characteristic for acidic polysaccharides in SE-PAGE or capsular structure in electron microscopy (Fig. 1, Table 1).

All five fish injected with *S. iniae* NUF631 died within 5 days post-inoculation, while none of the fish injected with the mutants, except for strain 11–19, died during the observation period. Strain 11–19 killed two of five injected fish at 6 and 7 day post-inoculation; from the dead fish *S. iniae* of K^- type were reisolated (Table 1).

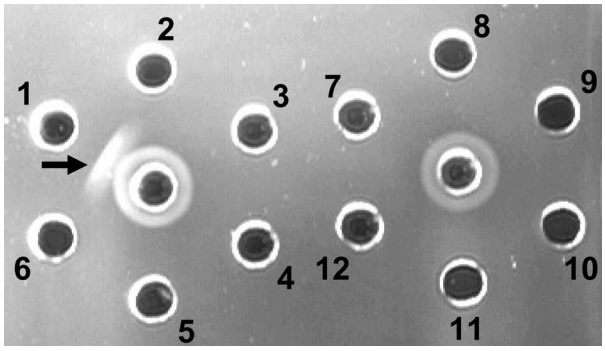

Fig. 1. Immunodiffusion of autoclave-extracted antigens from the NUF631 and mutant strains. 1, NUF631; 2, 4–58; 3, 4–79; 4, 4–94; 5, 6–20; 6, 9–16; 7, 10–15; 8, 10–86; 9, 11–19; 10, 11–34; 11, 11–36; 12, 13–94. Center well, rabbit anti-NUF631 serum; arrow, precipitation line due to K^+ type-specific antigen.

Table 1. Deletion of K^+ type–specific traits in the mutant strains derived from *S. iniae* NUF631 and virulence in Japanese flounder

Mutant strain	SA*1	AP*2	Capsule	Virulence*3	Recovery of K^- type*4 cells from survivor
NUF631	+	+	+	5/5	–
4–58	–	–	–	0/5	2/5
4–79	–	–	–	0/5	2/5
4–94	–	–	–	0/5	2/5
6–20	–	–	–	0/5	0/5
9–16	–	–	–	0/5	0/5
10–15	–	–	–	0/5	1/5
10–86	–	–	–	0/5	3/5
11–19	–	–	–	2/5	3/3
11–34	–	–	–	0/5	1/5
11–36	–	–	–	0/5	0/5
13–94	–	–	–	0/5	2/5

*1SA, K+type–specific antigen; *2AP, acidic polysaccharides; *3number of dead fish/number of challenged fish; *4, number of K^- type recovered fish/number of survivor.

Southern hybridization with DIG-labeled Tn-1 probe revealed one band in each mutant, suggesting that single copy of Tn*916* was inserted into the mutant chromosome (Fig. 2). To study the Tn*916*-inserted sites, nucleotide sequences of chromosomal regions adjacent to Tn*916* were determined, and the homologies of their deduced amino acid sequences were examined with database (Table 2). Six mutants (strains 6–20, 9–16, 10–15, 11–34, 11–36 and 13–96) had high homologies with proteins implicated in *S. iniae* capsule synthesis (Cps). Three mutants, strains 4–58, 4–79 and 11–19, had relatively high homologies with CitG (dephospho-CoA triphosphoribosyl transferase), MtlA (mannitol-specific enzyme II of the phosphotransferase system) and adenylosuccinate synthetase, respectively, enzymes of basic metabolic functions. The rest two, strains 4–94 and 10-86, had homologies with proteins of putative regulatory function and unknown function, respectively.

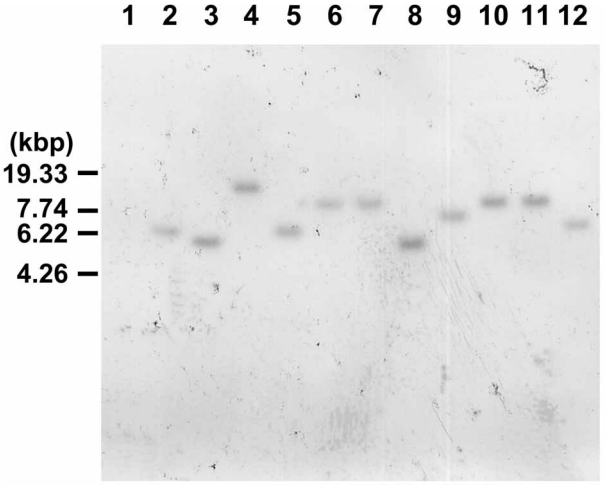

Fig. 2. Southern hybridization of *Hin*dIII fragments of chromosomal DNAs from *S. iniae* strains with a Tn916 probe (Tn-1). No.1 to12 are the same as Fig. 1.

Table 2. Homologous proteins to the sequences at Tn916 insertion sites of the *S. iniae* mutant chromosomal DNA

Mutant strain	Homologous protein	Organism	Putative function	Accession number	% Identity */no. of amino acid
4–58	CitG	*S. mutans*	Citrate metabolism	NP_721406	55/152
4–79	MtlA	*S. pyogenes*	Mannitoltransport	AAT87106	83/154
4–94	Sensor histidine kinase	*S. agalactiae*	Transcription regulator	NP_689112	68/353
6–20	CpsH	*S. iniae*	Capsule synthesis	AAY17300	99/340
9–16	ORF276	*S. iniae*	Capsule synthesis	AAY17304	96/133
10–15	CpsH	*S. iniae*	Capsule synthesis	AAY17300	96/169
10–86	CBS domain containing protein	*S. pyogenes*	Unkown	AAT86467	79/117
11–19	Adenylosuccinate synthetase	*S. pyogenes*	Purinemetabolism	AAZ50755	88/59
11–34	CpsH	*S. iniae*	Capsule synthesis	AAY17300	100/104
11–36	CpsM	*S. iniae*	Capsule synthesis	AAY17310	97/140
13–96	CpsI	*S. iniae*	Capsule synthesis	AAY17301	97/96

* Identities were calculated by BLASTX program at the National Center for Biotechnology Information (NCBI).

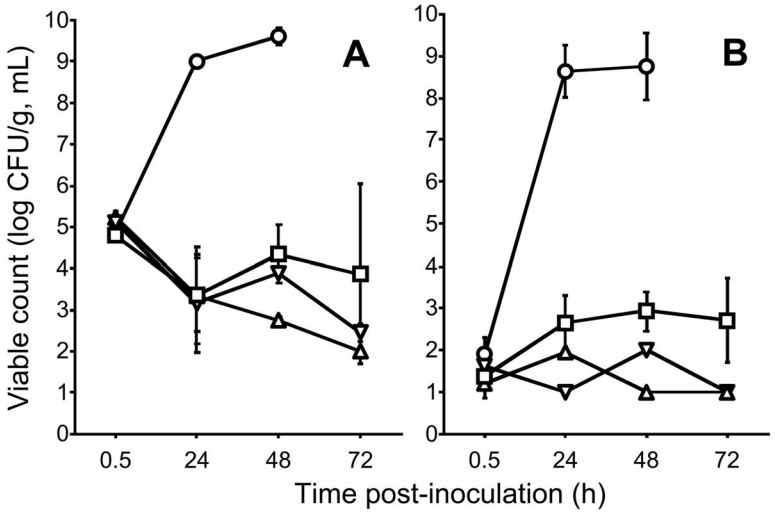

Fig. 3. Changes in viable count of *S. iniae* strains in the kidney (A) and blood (B) of Japanese flounder. Bacterial suspension (1×10^6 CFU/mL) was intravenously inoculated at 0.1 mL/100 g body weight. Bars represent standard deviations (n = 3). ○, NUF631; △, 6−20; ▽, 9−16; □, 11−36.

Fate of S. iniae *NUF631 and mutant strains in Japanese flounder and plasma*

Japanese flounder were intravenously inoculated with either NUF631 or mutant strains at a dose of 10^5 CFU/100 g body weight. At 30 min post-inoculation, almost the same level of viable count as the inoculated dosage was detected in the kidney of both NUF631- and mutant-inoculated flounder. After that, viable count of NUF631 in the kidney increased significantly, and all fish died by 72 h post-inoculation. On the other hand, those of mutant strains decreased (Fig. 3A). In the blood, viable count of NUF631 increased significantly as in the kidney, whereas those of mutant strains were kept at low levels for 72 h (Fig. 3B). Both NUF631 and mutant strains grew in plasma *in vitro* at almost the same rate (Fig.4).

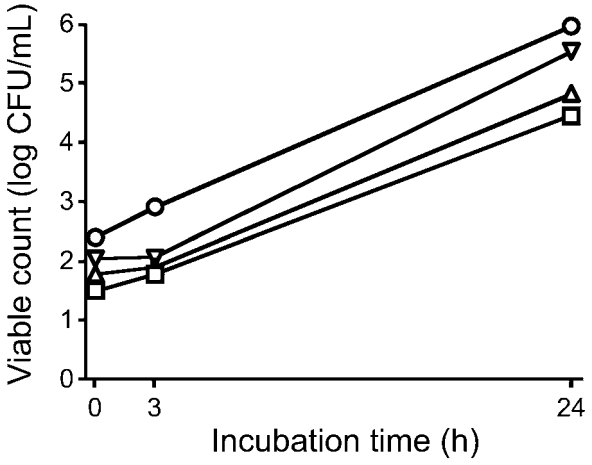

Fig. 4. Changes in viable count of *S. iniae* strains in flounder plasma. ○, NUF631; △, 6−20; ▽, 9−16; □, 11−36.

Bacterial cell surface hydrophobicity

The percentage of bacteria partitioned to hydrocarbon phase was 8.2% for NUF631, and 78.9%, 85.1% and 86.3% for mutant strains 6−20, 9−16 and 11−36, respectively (Fig. 5). These results indicate that the cell surface of NUF631 is more hydrophilic than mutant strains.

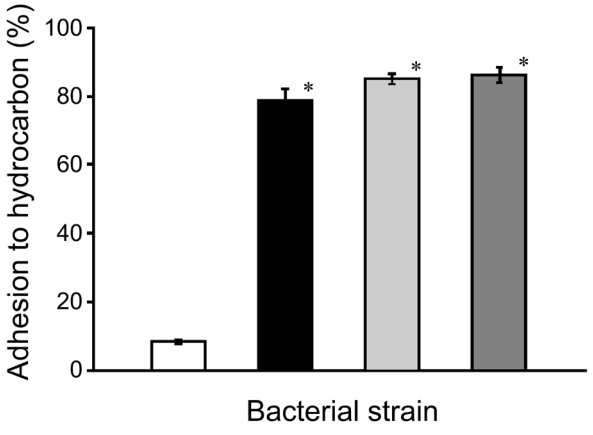

Fig. 5. Hydrophobicity of the *S. inia*e strains. The percentage of partitioning in the hydrocarbon phase was calculated with the following formula: 100 × [A660 of initial bacterial suspension − A660 of hydrocarbon-treated aqueous phase]/ A660 of initial bacterial suspension, where A660 = absorbance at 660 nm. Values that are significantly different ($P < 0.01$) from the NUF631 value are indicated by an asterisk. □, NUF631; ■, 6-20; ▒, 9−16; ■, 11−36.

Resistance to phagocytic and bactericidal activities of peritoneal macrophages

The phagocytic rate of macrophages for NUF631 was 9.7%, whereas those for mutant strains 6−20, 9−16 and 11−36 were 39.8%, 43.4% and 37.3%, respectively.

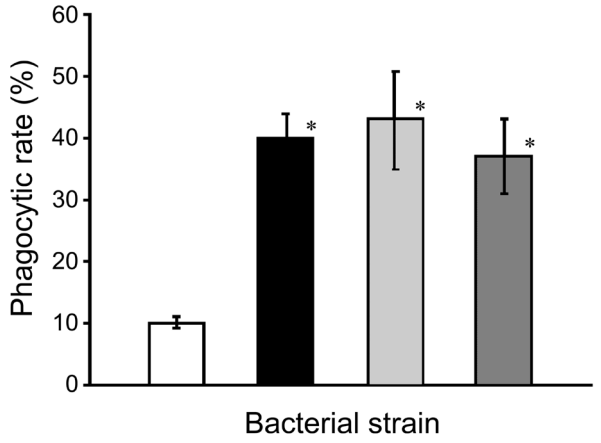

Fig. 6. Phagocytosis of the *S. iniae* strains by flounder peritoneal macrophages. Values that are significantly different ($P < 0.01$) from the NUF631 value are indicated by an asterisk. □, NUF631; ■, 6–20; ▫, 9–16; ▪, 11–36.

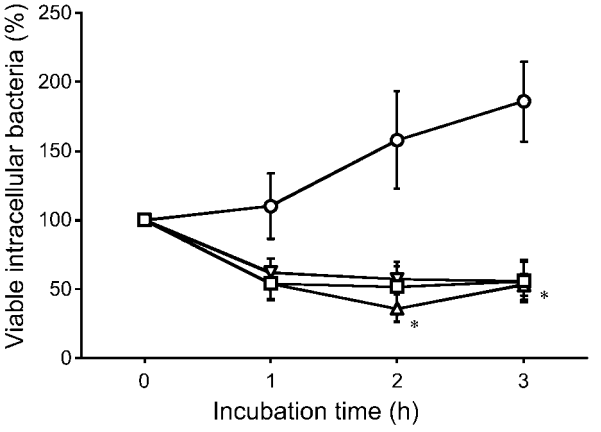

Fig 7. Intracellular survival of *S. iniae* strains within flounder peritoneal macrophages. Values that are significantly different ($P < 0.01$) from the NUF631 value are indicated by an asterisk. ○, NUF631; △, 6–20; ▽, 9–16; □, 11–36.

Mutant strains were phagocytosed more efficiently than NUF631 (Fig. 6). Changes in viable count of intracellular bacteria are shown in Fig. 7. Viable count of NUF631 continued to increase for 3 h, whereas those of mutant strains decreased.

Chemiluminescence response of peritoneal macrophages

Chemiluminescence response, indicating the production of reactive oxygen species (ROS), was observed in mutant strains accompanied with peaks at 4 to 5 min post-stimulation, whereas in NUF631 such response was almost negligible (Fig. 8).

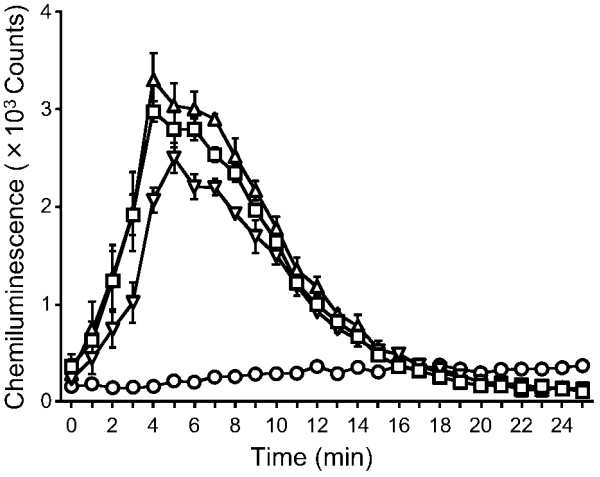

Fig 8. Chemiluminescence response of flounder peritoneal macrophages to the *S. iniae* strains opsonized with flounder normal serum. ○, NUF631; △, 6–20; ▽, 9–16; □, 11–36.

Discussion

Kanai *et al*. (2006) reported that *S. iniae* strains isolated in Japan were serologically divided into two groups, designated K^+ type and K^- type, by agglutinating reactions with two kinds of anti-*S. iniae* serum and that cell surface acidic polysaccharides, a type-specific polysaccharide antigen and a capsule were detected in strains of K^+ type but not in those of K^- type. Furthermore, the authors showed that strains of K^+ type were virulent, whereas those of K^- type were avirulent. In this study transposon mutagenesis was used to clarify the relatedness of these properties. As a result, all the mutant strains that transformed from K^+ type to K^- type have lost the productivities of the cell surface acidic polysaccharides, K^+ type-specific antigen and capsule, and their virulence were highly attenuated. These results strongly indicate that the K^+ type-specific antigen is the cell surface acidic polysaccharides and a component of the capsule and that they play a crucial role in *S. iniae* virulence.

Sequencing the chromosomal regions flanking Tn*916* of eleven mutant strains revealed that six of them, strains 6–20, 9–16, 10–15, 11–34, 11–36 and 13–96, were mutated by destruction of genes within a *S. iniae* capsule synthesis operon. In the other mutants Tn916 was inserted into genes encoding proteins that had homologies with proteins of basic metabolic functions or gene regulation (Table 2). Although these proteins might contribute to synthesis or construction of the capsule, it is likely that they also participate in other metabolic processes so that mutations other than capsule formation could have occurred. Accordingly, strains 6–20, 9–16 and 11–36 were chosen for the later experiments studying the roles of capsule in *S. iniae* virulence.

The result of bacterial viability test in flounder plasma suggests that *S. iniae* resists to bactericidal activities of humoral factors irrespective of the presence of the capsule (Fig. 4). On the other hand, uncapsulated mutant strains decreased and capsulated NUF631 increased in number in the kidney, indicating that cellular bactericidal mechanisms may participate in their clearance from the host and that the capsule may be one of factors to resist to such mechanisms (Fig. 3). A recent histological study indicated that infected *S. iniae* cells were rapidly taken up by renal or splenic macrophages and proliferated within them (Nguyen *et al*., 2001). Zlotkin *et al*. (2003) described 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. These phenomena were reproduced to some extent in *in vitro* experiment using flounder peritoneal macrophages and *S. iniae* cells opsonized with flounder normal serum. In the experiment, capsulated NUF631 cells resisted to phagocytic and bactericidal activities of peritoneal macrophages, whereas uncapsulated mutants were phagocytosed and intracellularly killed by macrophages, probably, partly due to the action of ROS produced upon phagocytosis (Figs. 6, 7 and 8).

Possible mechanisms that allow capsulated cells to evade the phagocytic activities would include their cell surface hydrophilicity, prevention of complement fixation and interference of the contact between opsonized cells and complement receptors on macrophages, etc. It was reported that hydrophobic bacteria were more easily phagocytosed by human neutrophil than hydrophilic ones (Absolom, 1988). So, it is reasonable to assume the involvement of hydrophilicity of capsulated *S. iniae* phenotype for evasion from phagocytosis. However, under the presence of opsonins (in the present case, normal serum), the latter two mechanisms would also participate in the evasion. Our unpublished data that mutant strains opsonized with heat-inactivated normal serum were less phagocytosed than those opsonized with intact normal serum would support such an opinion. It was also reported that both capsulated and uncapsulated pneumococci activated guinea pig complement via alternative pathway and that the C3b fixed to the cell wall irrespective of capsulation (Winkelstein *et al*., 1980). Further studies are needed to clarify whether this is applicable to the case of *S. iniae*.

Hirakata *et al*. (1993) suggested that several mechanisms may be involved in the elimination of intravenously administrated bacteria by tissue macrophages. In the present study, although *in vitro* phagocytosis experiment using peritoneal macrophages showed different phagocytic rates against capsulated and uncapsulated *S. iniae* cells, *in vivo* bacterial clearance test revealed that rapid entrapment of intravenously administrated bacteria by renal macrophages was observed for both phenotypes at almost the same rate (Fig. 3). So, different mechanisms between peritoneal and renal macrophages may participate in *S. iniae* phagocytosis.

In this study, reduced production of ROS was observed upon phagocytosis of capsulated *S. iniae* cells in comparison with uncapsulated ones. Similar observation on capsulated and uncapsulated cells was shown in *Erysipelothrix rhusiopathiae* (Shimoji *et al*., 1996). Decreased oxidative response of macrophages was observed upon opsonin-independent phagocytosis (Kobzik *et al*., 1990). It has been suggested that the receptors used upon phagocytosis influence the intracellular fate of bacteria (Ishibashi and Arai., 1990; Drevets *et al*., 1992). Although mechanisms for the prevention of ROS production are unclear, difference in receptors for phagocytosis of capsulated and uncapsulated *S. iniae* cells may influence the triggering of ROS formation cascade.

In conclusion, using transposon mutagenesis, we demonstrated that the *S. iniae* capsule plays a crucial role in the virulence through prevention of phagocytosis and ROS production by macrophages. It is also indicated that the K^+ type-specific antigen of *S. iniae* (Kanai *et al*., 2006) is the cell surface acidic polysaccharides and a component of the capsule. Further studies are needed to clarify the mechanisms by which the capsulated *S. iniae* prevents ROS production of macrophages for understanding the *S. iniae* pathogenicity.

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