Bull. Eur. Ass. Fish Pathol., 29(1) 2009, 16

Surface properties of *Streptococcus dysgalactiae* strains isolated from marine fish

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Abstract

The cell surface related properties, such as the hemagglutination capacity and hydrophobicity, of the *Streptococcus dysgalactiae* isolates recovered from amberjack (*Seriola dumerili*), yellowtail (*S. quinqueradiata*), and mullet species (*Mugil cepalus*) were examined. In addition, the relationship between these properties and the adhesion and the invasiveness of the isolates was investigated. The *S. dysgalactiae* isolates exhibited hydrophobic properties. Their hemagglutination activity was highest against amberjack and guinea pig erythrocytes followed by bovine, equine, and avian erythrocytes. The *S. dysgalactiae* isolates adhered to and invaded cells of a fish epithelial cell line cultured *in vitro*. It was observed that fish isolates of *S. dysgalactiae* had similar adhesion and possibly invasion properties with that of previous studies on *S. dysgalactiae* of bovine mastitis when examined in an appropriate cell line.

Introduction

Streptococcus dysgalactiae subsp. *dysgalactiae* is a Gram-positive bacterium, classified among Lancefield group C streptococci (Garvie et al., 1983). As a pathogen, this organism can cause both contiguous and environmental infections, depending on its distinctive distribution and adhesion ability (Bramly & Dodd, 1984). *S. dysgalactiae* infection has been associated with cutaneous lesions in humans (Fernández-Aceñero & Fernández-López, 2006), mastitis and subcutaneous cellulitis in cows (Seno & Azuma, 1983; Chénier et al., 2008). In 2002, an epizootic caused by the α-hemolytic Lancefield group C *S. dysgalactiae* broke out among cultured yellowtail (*Seriola quinqueradiata*) and amberjack (*S. dumerili*) populations in the southern parts of Japan, and the infected fish exhibited a typical form of necrosis in the caudal peduncle (Nomoto et al., 2004). The pathogen has been also isolated from grey mullet (*Mugil cephalus*) in Taiwan. Since their clinical signs were strongly similar, this epizootic was initially assumed to have been caused by a *Lactococcus garvieae* infection (Nomoto et al., 2004). Since then, several comparison studies have been performed for biochemical and genetic characterization of fish isolates and mammalian isolates of *S. dysgalactiae* (Nomoto et al., 2006; Nomoto et al., 2008). However, the virulence factors and infection mechanisms that characterize *S. dysgalactiae* as a fish pathogen remains unknown. Therefore, the quest for a vaccine against this pathogen is hindered by the lack of knowledge regarding the pathogenesis and virulence determinants of *S. dysgalactiae*. In this study, we aimed to evaluate some virulence determinants of *S. dysgalactiae* isolated from fish, such as its hemagglutination and hydrophobic

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properties. Furthermore, we investigated the ability of this pathogen to adhere to and invade piscine epithelial cells *in vitro*.

Materials and methods

Bacterial isolates

Seventeen isolates of *S. dysgalactiae*, isolated from diseased cultured fish from the southern parts of Japan between 2002 and 2006, were investigated in this study (Table 1). Four Gram-positive *L. garvieae* isolates from yellowtail (*S. quinqueradiata*) and jack mackerel (*Trachurus japonicus*) (Table 1) were also included in some of the tests for comparison. *Escherichia coli* DH5α (Toyobo Co., Ltd., Japan) was used as a negative control in the invasion experiment (Almeida & Oliver, 1995).

Growth conditions and bacterial identification

Streptococcus dysgalactiae and *L. garvieae* isolates were routinely cultured at 24 h on Todd-Hewitt agar (THA; Difco, Michigan, USA). Stock cultures were maintained at –80°C in Todd-Hewitt broth (THB; Difco, Michigan, USA). *Escherichi coli* was grown in Luria-Bertani medium (LB; Bio 101, Inc., Calif., USA) at 37°C for 24 h. Using the Pastorex Strep® agglutination test (Bio-Rad, Marnesla-Coquette, France), we confirmed that the *S. dysgalactiae* isolates were of the Lancefield group C serotype (Lancefield, 1933). The *S. dysgalactiae* cells were identified using API 20 STREP® (bioMerieux, Marcy-l'Etoile, France) and by performing a polymerase chain reaction assay targeting the intergenic 16S–23S rDNA spacer region, which is specific to *S. dysgalactiae* (Nomoto et al., 2004).

Surface hydrophobicity

The cell-surface hydrophobicity of the *S. dysgalactiae* and *L. garvieae* isolates was evaluated by performing the microbial adhesion to hydrocarbons (MATH) assay according to the procedure described by Rosenberg et al. (1980), with some modifications. In brief, the bacteria were grown in THB at 37°C overnight. The bacterial cells were harvested by centrifugation at 2,190 g for 20 min. The harvested cells were then washed twice with 0.1 M potassium phosphate buffer (pH 7.0), resuspended in the same buffer and the absorbance (initial optical density) was measured at 400 nm. A small amount of *n*-octane (0.4 ml) was mixed with the cell suspension (2.4 ml) for 2 min using a vortex mixer. The mixture was allowed to stand for 15 min, following which the aqueous phase was carefully transferred to a 1-ml cuvette and the absorbance was measured at 400 nm. The result was expressed as the percentage of the optical density recorded for the aqueous phase against the initial optical density.

Hemagglutination activity (HA)

The ability of *S. dysgalactiae* isolates to agglutinate various erythrocytes was determined by using the method proposed by Izumi et al. (2005). Guinea pig, avian, bovine, equine (Nippon Bio-Test Laboratories, Japan) and amberjack erythrocytes were used in this study. The bacterial cells of all *S. dysgalactiae* and *L. garvieae* isolates were incubated in THB for 16 h at 37°C, harvested by centrifugation at 2,190 g for 30 min, and washed twice with phosphate-buffered saline (PBS; pH 7.2). The cell concentration was adjusted to 1×10^8 colony forming unit per ml (cfu/ml) by using

	Isolate no.	Source	Fish	Year of isolation
Lancefield group C	12-06	Kagoshima	Amberjack	2002
Streptococcus dysgalactiae	Kdys0420	Kagoshima	Amberjack	2004
	Kdys0429	Kagoshima	Amberjack	2004
	Kdys0435	Kagoshima	Amberjack	2004
	Kdys0439	Kagoshima	Amberjack	2004
	Kdys0520	Kagoshima	Amberjack	2005
	Kdys0536	Kagoshima	Amberjack	2005
	Kdys0603	Kagoshima	Amberjack	2006
	Kdys0606	Kagoshima	Yellowtail	2006
	KN041101	Kagoshima	Amberjack	2004
	KN06901	Kagoshima	Amberjack	2006
	KC03913	Kochi	Amberjack	2003
	NM03920	Kochi	Amberjack	2003
	SK061005	Kochi	Amberjack	2006
	KU05801	Miyazaki	Amberjack	2005
	94455	Taiwan	Mullet	2005
	94485	Taiwan	Mullet	2005
Lactococcus garvieae				
Non-capsulated	SS9034	Nagasaki	Yellowtail	1990
	NSS9310	Nagasaki	Yellowtail	1993
Capsulated	KT06A1	Miyazaki	Jack mackerel	2006
	EH05903	Nagasaki	Yellowtail	2005

Table 1. *Streptococcus dysgalactiae* and *Lactococcus garvieae* isolates included in this study.

a spectrophotometer operating at 400 nm. Serial 2-fold dilutions of the cell suspension (25 µl/well) were prepared in 96-well U-bottom microtiter plates (Sankyo Co., Tokyo, Japan) and guinea pig, avian, bovine, or equine erythrocytes, all of which had been adjusted to a concentration of 2% in PBS, while amberjack erythrocytes were 1%, was added to each well (25 µl/well). After mildly stirring the mixtures for 20 s, the plates were incubated at 25°C for 1 h and then at 4°C for 4 h. This assay has been applied on static and shaking cultures, or before and after washing of bacterial cells with PBS. HA assay was performed at least twice for each isolate.

Adherence assay

The adherence capacity of only *S. dysgalactiae* and *L. garvieae* isolates was evaluated *in vitro* according to the method proposed by Calvinho & Oliver (1998), with some modifications. Epithelioma papillosum cells (EPCs) of the carp (*Cyprinus carpio)* (Wolf & Mann, 1980) were used in this assay. The EPCs were grown in monolayers on 24-well tissue culture plates (Costar, Corning, Inc., NY, USA) in Leibovitz-15 (L-15) medium (Gibco Invitrogen, USA) containing 10% (v/v) fetal bovine serum and penicillin (5 µg/ml; Sigma-Aldrich Inc., USA), incubated at 25°C, and inspected daily until they attained 90%

confluency. Initially, The bacterial cells of all S. dysgalactiae and L. garvieae isolates were incubated in THB overnight at 37°C to mid-logarithmic phase (ca. 10^8 cfu/ml), harvested by centrifugation at 2,190 g for 30 min, and washed twice with phosphate-buffered saline (PBS; pH 7.2), and serially diluted 10-fold were performed to adjust inoculums nearly 10^5 cfu/ml, after that the exact number of inoculums bacteria was estimated by the spread-plate method performed using THA. The procedure employed to measure the number of bacteria adhering to the EPCs comprised the following steps: (1) The plates were carefully washed three times with the L-15 medium. (2) A fixed volume (900 µl) of antibiotic-free L-15 medium containing 2.5% (v/v) fetal bovine serum was added to each plate. (3) The bacterial suspension (100 µl) was added to each well, the concentration of each isolate in Table 2. (4) The plates were centrifuged at 570 g for 15 min. (5) The plates were incubated at 25°C for 90 min. (6) The plates were carefully washed six times at 25°C with the antibiotic-free L-15 medium to remove the non-adherent bacteria. (7) The EPCs were lysed with 200 µl of 0.25% (v/v) Triton® X-100 (MP Biomedicals, USA) in PBS, with vigorous stirring for 15 min. (8) To each well, 800 µl of PBS was added and mixed thoroughly. (9) Finally, the bacterial suspension thus obtained was serially diluted 10-fold, and the number of adherent bacteria was estimated by the spread-plate method performed using THA. Thus, the total number of bacteria that adhered to the EPC surfaces and those that were internalized by the cells was quantified.

Invasion assay

The invasion assay was performed according to the method described by Almeida & Oliver (1995), with some modifications. The bacterial invasion assay was performed using standard in vitro methodology involving incubation of bacteria with epithelial cells followed by selective killing of extracellular bacteria by gentamicin and penicillin G and release of internalized bacteria by lysis of EPCs with detergent (Almeida & Oliver, 1995). Five isolates of *S. dysgalactiae* were selected on the basis of their hydrophobic and adhesion properties, and *E. coli* was used as a negative control. The EPCs were cocultured with *S. dysgalactiae* or *E. coli* by using the procedure described above for the adherence assay. After the plates were centrifuged at 570 g for 15 min, the cocultures were incubated at 25°C for 30 min and then the culture supernatants were discarded and the plates were carefully washed three times with the L-15 medium, and an L-15 medium containing penicillin (5 µg/ml; Sigma-Aldrich Inc., USA), gentamycin (100 µg/ml, Sigma-Aldrich Inc., USA), and 2.5% (v/v) fetal bovine serum was added then the plates were re-incubated for 90 min. Before the experiment, the efficiency of the antibiotics against *S. dysgalactiae* was tested. After the L-15 medium supplemented with antibiotics was removed from the EPC surface, the EPCs were washed three times with the antibiotic-free L-15 medium. The EPCs were then lysed, plated in triplicate on THA, as described in adhesion assay, and then the bacterial concentration (cfu/well) was determined.

Statistical analysis

All the tests except HA assay were performed in triplicate. The results were expressed as

Isolates	Inoculum size (cfu/well)	Percent adhesion
12-06	3.0×10^4	114.0 ± (8.5)b
Kdys0420	14.0×10^4	68.9 ± (7.7)b
Kdys0429	10.0×10^4	88.0 ± (11.5)b
Kdys0435	32.0×10^4	86.4 ± (6.8)b
Kdys0439	2.4×10^4	104.1 ± (12.0)b
Kdys0520	4.0×10^4	91.7 ± (11.0)b
Kdys0536	13.0×10^4	98.1 ± (2.0)b
Kdys0603	2.2×10^4	117.8 ± (22.3)b
Kdys0606	9.0×10^4	59.5 ± (3.5)b
KN041101	2.2×10^4	110.5 ± (1.5)b
KN06901	1.4×10^4	105.3 ± (2.7)b
KC03913	12.9×10^4	113.2 ± (17.1)b
NM03920	1.2×10^4	96.9 ± (9.8)b
SK061005	1.0×10^4	120.0 ± (5.7)b
KU05801	4.0×10^4	123.3 ± (11.7)b
94455	9.0×10^4	66.8 ± (18.3)b
94485	25.0×10^4	56.4 ± (3.7)b
*SS9034	2.2×10^5	6.4 ± (2.5)a
*NSS9310	3.1×10^5	2.1 ± (0.6)a
*KT06A1	4.2×10^5	3.2 ± (2.0)a
*EH05903	4.0×10^4	4.4 ± (1.2)a

Table 2. Percent adhesion values of *Streptococcus dysgalactiae* and *Lactococcus garvieae* to epithelium papillosum cells (EPC) expressed as the mean ± SEM. The initial inoculum size is expressed in numbers of colony forming units per well (cfu/well). Percent adhesion values followed by same letter are not significantly different, and percent adhesion values followed by different letters are significantly different ($p < 0.01$, one-way ANOVA, followed by Tukey's test).
* *L. garvieae* strains.

mean ± standard error of the mean (SEM), and were statistically analyzed by one-way analysis of variance (ANOVA; Tukey compromise test, $P<0.01$).

Results

Surface hydrophobicity

The *S. dysgalactiae* isolates exhibited hydrophobic properties. Their percentage adherence to *n*-octane ranged from 73.9 ± 8.2% to 91.6% ± 2.7%. Statistically, the hydrophobic value of *S. dysgalactiae* isolate 94485 (53 ± 10.9%) was significantly lower than that of all the other *S. dysgalactiae* isolates, except for Kdys0439 (73.9 ± 8.2%) ($P < 0.01$; ANOVA). On the other hand, the hydrophobic values of *L. garvieae* isolates (1.3± 1.0% to 5.6± 1.2%) were significantly lower than those of all the *S. dysgalactiae* isolates ($P < 0.01$; ANOVA).

	Inoculum size (cfu/well)	30 min (cfu/well)	90 min (cfu/well)
12-06	3.9×10^7	$1.6 \pm (0.3)\times 10^3$	$2.0 \pm (0.3)\times 10^3$
Kdys0603	2.5×10^7	$1.6 \pm (0.6)\times 10^3$	$1.7 \pm (0.6)\times 10^3$
Kdys0606	4.0×10^7	$0.6 \pm (0.1)\times 10^2$	$1.0 \pm (0.2)\times 10^2$
SK061005	1.6×10^7	$1.9 \pm (0.6)\times 10^3$	$2.1 \pm (0.9)\times 10^3$
94485	2.8×10^7	$0.5 \pm (0.1)\times 10^2$	$0.6 \pm (0.3)\times 10^2$

Table 3. Recovery of intracellular *Streptococcus dysgalactiae* bacteria from infected epithelioma papillosum cells (EPC) after 30 and 90 min incubation. The recovery bacteria are expressed as the mean ± SEM in terms of number of colony forming units per well (cfu/well).

Hemagglutination

All the *S. dysgalactiae* isolates exhibited similar hemagglutination values. The highest hemagglutination value was recorded against amberjack and guinea pig erythrocytes ($1:2^4$) while the hemagglutination values were $1:2^2$, $1:2^2$, and $1:2^1$ against the bovine, equine, and avian erythrocytes, respectively. Hemagglutination titres of Taiwanese isolates against amberjack erythrocytes were $1:2^3$. No difference was observed in the hemagglutination values either between the static and shaking cultures or before and after the vigorous washing of the bacterial cells with PBS. *L. garvieae* did not exhibit agglutination with any of the types of erythrocytes tested.

Adherence and invasion assays

The *S. dysgalactiae* isolates strongly adhered to the EPCs *in vitro*, and the percent adhesion values ranged from 56.4% ± 3.7% to 123.3% ± 11.7% (Table 2). On the other hand, the percent adhesion values of the *L. garvieae* isolates were significantly lower than that of all the *S. dysgalactiae* isolates ($P < 0.01$; ANOVA). The *S. dysgalactiae* isolates apparently invaded the EPCs *in vitro*, while *E. coli* did not, and the invasion was detected 30 min after the inoculation (Table 3).

Discussion

Recently, *S. dysgalactiae* has been found to cause mortality with severe necrotic lesions in the caudal peduncle among farmed fish in Japan (Nomoto et al., 2004). However, no information is available on the initial step of fish-pathogen interaction. In addition, the strong clinical similarity between two pathogens, *S. dysgalactiae* and *L. garvieae*, with regard to infection mechanisms and virulence factors was not discussed yet. The hemagglutination assay has been used to evaluate the adhesion and hemagglutination of various Gram-positive bacteria (Izumi et al., 2005). It is noteworthy that although both *S. dysgalactiae* and *L. garvieae* exhibit fimbria-like surface structures (Ooyama et al., 2002; Nomoto et al., 2004), all *S. dysgalactiae* isolates included in this study exhibited agglutination with amberjack and guinea pig erythrocytes, but *L. garvieae* isolates did not. This result revealed that the fimbria-like structures of *S. dysgalactiae* may be responsible for its hemagglutination activity. However, the role of these structures is unknown in the case of *L. garvieae*. These findings concur with those reported by Ooyama et al. (2002). The surface hydrophobicity of bacteria has been considered a primary indicator for evaluating

the capacity of bacteria to adhere to both animate and inanimate surfaces (Rosenberg et al., 1980; Romalde et al., 2000). In this study, the MATH assay revealed that the *S. dysgalactiae* isolates exhibited hydrophobic characters. However, a contradictory result was obtained in case of *L. garvieae*. The hydrophobicity of the *S. dysgalactiae* isolate 94485 was significantly lower than of almost all other *S. dysgalactiae* isolates. This might have been due to interisolate variations and environmental factors. For many bacterial pathogens, adhesion to and internalization into the epithelial cells are essential virulence factors responsible for the initiation of infections (Hasty et al., 1992; Valentin-Weigand et al., 1988). The adhesion and internalization properties of *S. dysgalactiae* isolated from bovine mastitis have been investigated using bovine mammary epithelial cells (Calvinho et al., 1996; Calvinho & Oliver, 1998). In this study, the fish isolates of *S. dysgalactiae* exhibited high adherence to the EPCs, while the *L. garvieae* isolates did not satisfactorily adhere to the EPCs. This result was consistent with those of the MATH and hemagglutination assay in this study and with previous observations indicating that bacterial hydrophobicity affectes the adhesion of *S. dysgalactiae* to the surface of host cells (Calvinho et al., 1996). Therefore, surface hydrophobicity is proportionally correlated with high adhesion percentage, therefore, surface hydrophobicity seems to be an important factor for *S. dysgalactiae* for the establishment of an infection during the initial stages of host-pathogen interaction. The invasion assay demonstrated the possibility of *S. dysgalactiae* to invade EPCs *in vitro*. *Streptococcus dysgalactiae* invasion was detected 30 min after the inoculation. Since gentamycin is bacteriostatic, we also used the bactericidal antibiotic penicillin to enhance killing of extracellular bacteria. Internalization may protect the pathogen from the host defense responses and the action of antibiotics (Almeida & Oliver, 1995). Moreover, the invasion capacity may represent a potentially important feature in the pathogenesis of *S. dysgalactiae* infection. However, the number of intracellular bacteria recovered was not proportional to the inoculum size, suggesting that a finite number of *S. dysgalactiae* were able to invade at a particular time. These findings concur with those reported by Rubens et al. (1992), who found that infected monolayers incubated with antibiotic-containing medium for up to 8 h contained a similar number of streptococci as at 2 h. These findings will help in the understanding of the pathogenesis of *S. dysgalactiae* infection in fish. The present study demonstrated that despite the differences in biochemical and genetic properties of mammalian and fish isolates of *S. dysgalactiae* (Nomoto et al., 2006; Nomoto et al., 2008), the adhesion and internalization ability of fish isolates were found to be similar with that of the previous studies on bovine isolates when examined in appropriate cell lines (Almeida & Oliver, 1995). The clinical symptoms of *S. dysgalactiae* and *L. garvieae* infections were similar among the infected fish. However, two pathogenic species differed in their putative virulence factors. The presence of a capsule enabled *L. garvieae* to evade phagocytic cells (Ooyama et al., 1999). On the other hand, adhesion to and internalization into the host cells appeared to be the mode of virulence in the case of *S. dysgalactiae*. Further studies

involving transmission electron microscope investigations of the possible mode of entry and the survival mechanisms of *S. dysgalactiae* in different types of eukaryotic cells are currently underway.

Acknowledgement

This study was partially supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Culture and Sports, Japan (19580212).

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