Phenotypic variation associated with an anti-phagocytic factor in the bacterial fish pathogen *Enterococcus seriolicida*

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ABSTRACT: *Enterococcus seriolicida* is the causative agent of an enterococcal infection in yellowtail *Seriola quinqueradiata* and is antigenically classified into 2 phenotypes, KG+ and KG–. Phenotypic variation from KG– to KG+ occurs readily on an artificial medium. The surface morphologies of the KG– and KG+ phenotypes were differentiated by scanning electron microscopy. KG– cells were more hydrophilic than KG+ cells and were resistant to phagocytosis by yellowtail head kidney phagocytes. The chemiluminescent response of these phagocytic cells was lower with the KG– phenotype than with the KG+ phenotype. The immune responses of yellowtail following injection of the 2 phenotypes differed: higher agglutinating titers were obtained with the KG+ phenotype compared to the KG– phenotype. Thus, the cell surface may function as an anti-phagocytic factor in *E. seriolicida* and apparently affects its immunogenicity.

KEY WORDS: *Enterococcus seriolicida* · *Seriola quinqueradiata* · Phagocytosis · Chemiluminescence · Immune response

INTRODUCTION

Non-haemolytic streptococcal infection is one of the most common diseases in yellowtail *Seriola quinqueradiata* cultured in Japan (Austin & Austin 1993). This infection has also been reported in the Japanese eel *Anguilla japonica* (Kusuda et al. 1978). Formerly, the causative agent of this disease was classified as *Streptococcus* sp., but recently it has been reclassified as *Enterococcus seriolicida* (Kusuda et al. 1991). Kitao (1982) reported that *E. seriolicida* could be serologically divided into 2 types, the so-called KG– and KG+ phenotypes, but the biochemical characteristics of the 2 phenotypes were identical. This antigenic variation arose after subculturing fresh isolates on *Streptococcus* agar (KF *Streptococcus* agar; Difco Laboratories, Detroit, MI,

USA) containing 2,3,5-triphenyltetrazolium chloride (TTC) or by successive subculturing on Todd Hewitt agar (TH agar; Difco). KG– phenotypes are so designated because, while they are agglutinated by anti-KG– serum, they are not agglutinated by antisera to the KG+ strain (KG 7904 KG+ phenotype strain). In contrast, KG+ strains can be agglutinated with both antisera to both KG+ and KG– phenotypes (Kitao 1982, 1993). Thus Kitao suggested that an envelope-like substance surrounding KG– cells inhibited the agglutination with the anti-KG+ serum. The KG– phenotype (fresh isolate) strain is more highly pathogenic in yellowtail than the KG+ phenotype (Kitao 1982).

Virulence factors of the causative agent are still unknown in spite of the importance of this disease in aquaculture. In the present study, we examined the cell surface properties of both phenotypes of *Enterococcus seriolicida*. We also studied the immune responses of yellowtail injected intraperitoneally with the 2 phenotypes.

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MATERIALS AND METHODS

Bacterial strains. *Enterococcus seriolicida* strains isolated from diseased yellowtails in Japan are listed in Table 1. Stock cultures were maintained at −70°C in 15% (vol/vol) glycerol-Todd Hewitt broth (THB; Difco). The bacteria were cultured in THB for 24 h at 30°C. The phenotype was confirmed by rabbit antiserum raised against KG+ cells. KG+ cells were obtained following 3 passages of the KG− phenotype on KF *Streptococcus* agar (Difco) and were confirmed as such using KG+ antiserum.

Preparation of anti-*Enterococcus seriolicida* KG− or KG+ phenotype rabbit antiserum. Cultures of both KG− and KG+ strains were killed by the addition of 0.3% formaldehyde (final concentration). The cells were washed with 0.85% saline solution, and resuspended in saline to an optical density of 1.0 at 620 nm. The cell suspension was emulsified in an equal volume of Freund's complete adjuvant (FCA) and 1 ml was inoculated in rabbits intramuscularly 3 times at 2 wk intervals. Blood was withdrawn and allowed to clot at 4°C for 1 d. Serum was harvested and kept at −80°C until required. Sera with a titer over 1:1240 against the homologous phenotype were used for the experiments.

Fish. The yellowtail used in this study were supplied by the Miyazaki Experimental Fisheries Station, Miyazaki, Japan. The mean body weight was 400 g and they were kept in 3000 l tanks with seawater thermoregulated at 23°C.

Electron microscopy. Both phenotypes of *Enterococcus seriolicida* NG8206 were cultured in THB at 30°C for 24 h. Bacteria were fixed overnight with a final concentration of 2% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.2). After fixation, the cells were washed 5 times with the same buffer. Cells were dehydrated in increasing acetone concentrations from 30% to 100%. They were subjected to critical point drying and then coated with a

layer of platinum palladium. Samples were examined with a scanning electron microscope (JEOL, JSM-35C, Tokyo, Japan) at an accelerating voltage of 25 kV

Bacterial adherence to hydrocarbon (BATH). The cell surface hydrophobicity was determined by the method of Rosenberg et al. (1980). Bacterial cultures were harvested, washed twice in Hanks' balanced salt solution (HBSS; Nissui), and suspended in this buffer at an optical density of 0.6 at 660 nm. Three ml of bacterial suspension were layered with 1 ml of n-octane in glass tubes. After incubation at 37°C for 20 min, specimens were agitated vigorously for 2 min and allowed to stand for 15 min to allow separation. The percent of partitioning in the hydrocarbon phase was calculated as

$$\frac{A_{660}(\text{bact. susp.}) - A_{660}(\text{hydrocarbon} - \text{treated bact. susp.})}{A_{660}(\text{bact. susp.})} \times 100$$

where A_{660} = absorbance at 660 nm.

Hemagglutination test. Hemagglutination was examined with live or formalin-killed bacterial suspensions. Both phenotypes were harvested, washed 5 times with physiological saline, and killed by adding formaldehyde to a final concentration of 0.3%. These suspensions were washed 3 times with HBSS and then adjusted to an optical density of 1.0 at 620 nm in HBSS. Bacterial suspensions were tested for hemagglutination of sheep red blood cells (SRBC), horse red blood cells (HRBC), rabbit red blood cells (RRBC) or yellowtail red blood cells (YRBC) by reacting 50 μl of bacterial suspension with 50 μl 2% (v/v) erythrocytes on a glass slide in a moist chamber. Strains were considered negative if visible agglutination did not occur within 10 min (Toranzo et al. 1983).

Opsonization of bacterial cells. Equal volumes of each bacterial suspension (1.0 × 10⁶ cells ml⁻¹) and yellowtail normal serum were mixed and incubated at 4°C for 60 min. After incubation, the bacterial cells were washed 3 times with HBSS. Opsonized bacterial cells were resuspended in HBSS and adjusted to an optical density of 0.6 at 620 nm.

Chemiluminescence assay. Kidney leucocytes from apparently healthy yellowtail were used in this study. The purification of the head kidney leucocytes was done according to Yoshida & Kitao (1991). The head kidney leucocytes from 3 fish were mixed. Viable phagocytic cells were determined by trypan blue exclusion staining; the cells were resuspended to a concentration of 6.0×10^6 cells ml⁻¹ in HBSS. The chemiluminescent assay was performed as described by Yoshida et al. (1993). Opsonized or non-opsonized phenotypes were used as the stimulant in the chemiluminescence assay. The chemiluminescence reactions were measured for 30 min by a TD4000M lumiphotometer (Laboscience) and then the peak values obtained with the KG− and KG+ phenotypes were compared.

Table 1. Strains of *Enterococcus seriolicida* used in this study and their properties

Strain	Host	Antiserum KG7409(−)	KG7409(+)	Phenotype
NG8206(−)	Yellowtail	+	−	KG−
NG8206(+)	Yellowtail	+	+	KG+
SA9201(−)	Yellowtail	+	−	KG−
SA9201(+)	Yellowtail	+	+	KG+
MZ9201(−)	Yellowtail	+	−	KG−
MZ9201(+)	Yellowtail	+	+	KG+
KG7409(−)	Yellowtail	+	−	KG−
KG7409(+)	Yellowtail	+	+	KG+

Adherence or uptake by phagocytes (phagocytosis). The phagocytosis assay was performed as described elsewhere (Kitao et al. 1991). Briefly, purified head kidney leucocytes were adjusted to 6.0×10^6 cells ml^{-1} in HBSS and allowed to adhere to a coverglass. The coverglass was rinsed 3 times with HBSS and overlayed with an *Enterococcus seriolicida* suspension. The suspension had been adjusted to 0.6 optical density at 620 nm in HBSS and had been opsonized with yellowtail serum. The coverglasses were incubated at 25°C for 2 h, rinsed with HBSS to remove free bacterial cells, and fixed with methanol. The cells were stained with Giemsa solution and the number of phagocytes containing or associated with more than 5 bacteria per 200 glass-adherent leucocytes was counted microscopically. All experiments were performed in triplicate.

Immune responses of yellowtail to the KG– and KG+ phenotypes. Bacterial cultures (NG8206) of each phenotypic strain were harvested, washed twice with 0.85% saline, and killed by adding formaldehyde to a final concentration of 0.3% at 4°C for 24 h. The bacteria were washed 3 times with physiological saline, and adjusted to an optical density of 1.0 at 620 nm. Equal volumes of bacterial suspension and FCA were emulsified. One ml of bacterin was injected intraperitoneally per yellowtail. Blood was withdrawn by cardiac puncture 4, 6, and 8 wk after the immunization. The agglutinating titer of each serum against the phenotypes (KG– and KG+) was examined by the microtiter agglutination assay (Roberson 1990).

Agglutinating titers in cultured fish. Blood samples were obtained from 42 farmed yellowtail (1500 to 2000 g) from a fish farm in Oita Prefecture, Japan, in 1993. Serum was obtained from the blood after clotting at room temperature and centrifugation at $1000 \times g$ for 10 min. The agglutinating titers of the serum samples for both phenotypes of *Enterococcus seriolicida* (KG– and KG+) were determined as mentioned above (Roberson 1990).

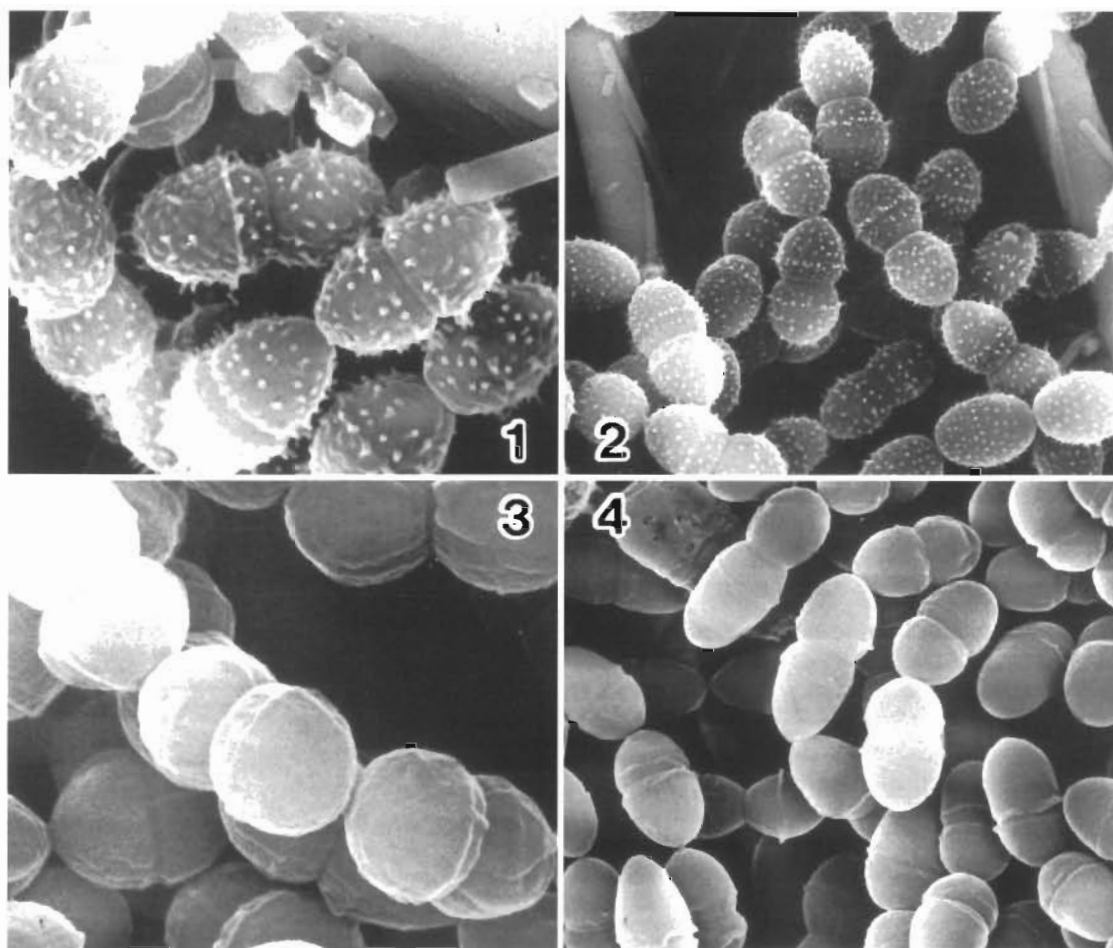**Statistical analysis.** Statistical significance was assessed using Student's *t*-test.

Fig. 1. *Enterococcus seriolicida*. Electron micrograph of KG– (1, 2) and KG+ phenotypes (3, 4) showing the difference in their cell surface morphologies. Magnifications are ×20000 (1, 3) and ×10000 (2, 4). KG+ phenotype cells showed smooth surface (3 and 4), while clumps of some material were observed on the surface of the KG– phenotype

RESULTS

Scanning electron microscopy. The scanning electron micrographs of *Enterococcus serioli-cida* KG+ and KG– phenotypes are shown in Fig. 1 The cell surface morphology of the KG+ phenotype was smooth, while KG– cells had a rough surface.

Serum agglutination. *Enterococcus seriolicida* KG+ was agglutinated with both rabbit anti-KG+ and anti-KG– phenotype antiserum. However, the KG– phenotype was agglutinated only with the rabbit anti-KG–phenotype antiserum.

Hydrophobicity of the cell surface. KG– phenotype strains partitioned into the hydrocarbon phase at rates of 1.0, 2.5, and 3.4%. In contrast, KG+ phenotype strains partitioned at rates of 23.4, 13.2, and 25.6%. Thus, the cell surface of the KG– phenotype was more hydrophilic than that of the KG+.

Hemagglutination. Neither formaldehyde-treated nor live cells of either phenotype agglu-tinated SRBC, HRBC, RRBC, or YRBC.

Chemiluminescence response. The chemilumines-cence responses of yellowtail head kidney leucocytes to both phenotypes is shown in Fig. 2. The chemilumi-nescence responses of yellowtail head kidney cells were higher with the opsonized KG+ phenotype than with the opsonized KG– cells. On the other hand, there was no significant difference in the chemiluminescence response between the non-opsonized phenotypes.

Phagocytosis/adherence. Phagocytic activity was higher with KG+ cells (40, 48, and 37%) than with KG– cells (20, 21, and 19%). The differences in these values were statistically significant ($p < 0.05$).
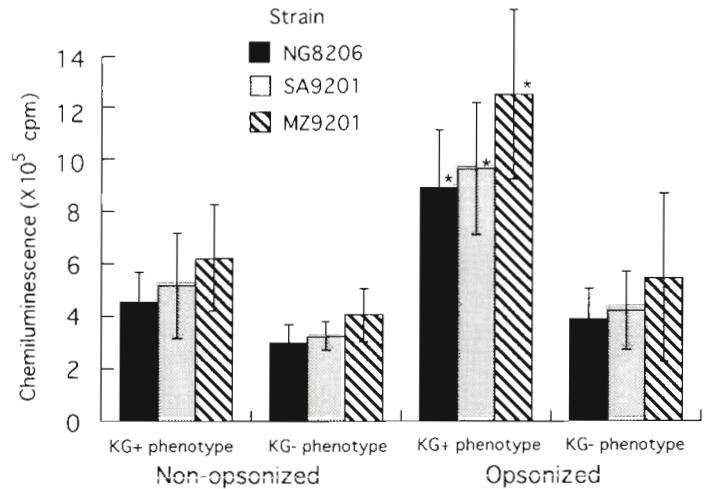

Fig. 2. Peak values of the chemiluminescent response of yellowtail *Seriola quinqueradiata* phagocytic cells against the KG– and KG+ pheno-types of *Enterococcus seriolicida*. The peak chemiluminescence values of phagocytic cells against opsonized KG+ phenotypes are significantly different from opsonized KG– phenotypes ($^*p < 0.05$). Error bars repre-sent standard deviations calculated from triplicate experments

Immune response of yellowtail to KG– or KG+ phenotypes. The serum agglutinating antibody titers of yellowtail injected with KG– or KG+ phenotypes are shown in Fig. 3. Serum agglutinating titers against KG+ cells from the fish injected with KG+ cells were high 4, 6, and 8 wk after the immunization. The agglutinating titers against the KG– phenotype strain were low throughout this period. The serum agglutinating titers against KG– phenotypes from the fish injected with KG– were lower than those against the KG+ phenotype strain 2 wk and 6 wk after the immunization ($p < 0.05$); how-ever, at 4 wk the difference was not significant.
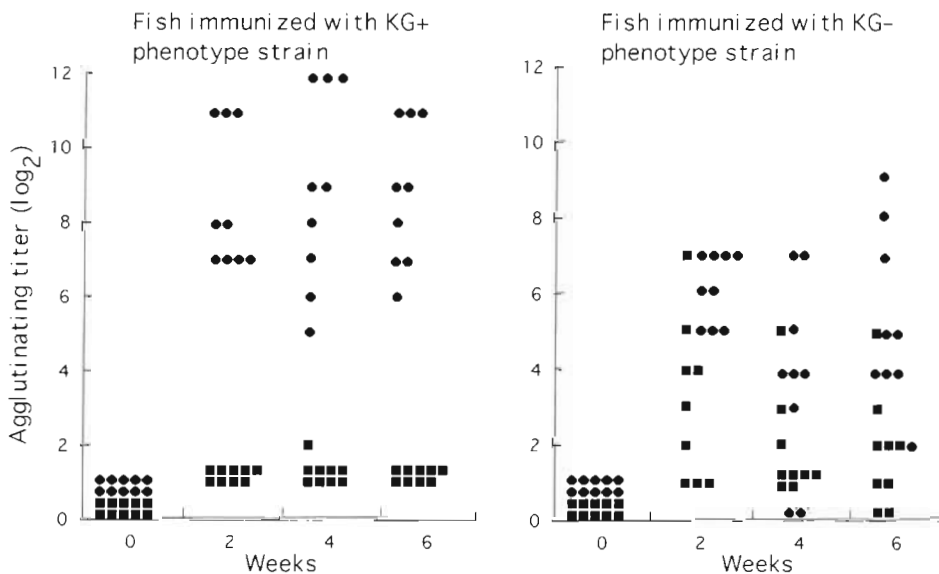

Fig. 3. Agglutinating titers against KG– (■) and KG+ (●) phenotype strains in sera from yellowtail *Seriola quinqueradi-ata* immunized with the KG– or KG+ phenotype strains of *En-terococcus seriolicida*. Fish in-fected with the KG+ phenotype produced high titers of ag-glutinating antibodies against the KG+ phenotype but not against the KG– pheno-type. The agglutinating titers against KG–phenotype strains from fish injected with KG– phenotypes were lower than those against the KG+ pheno-type strain at 2 and 6 wk after the immunization ($p < 0.05$)
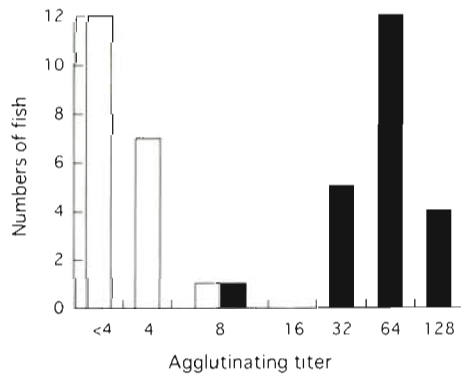
Fig. 4. Distribution of agglutinating titer against KG– phenotype (open bars) or KG+ phenotype (filled bars) of *Enterococcus seriolicida* in sera obtained from farmed yellowtail *Seriola quinqueradiata*

Agglutinating titers in cultured yellowtail. The distribution of agglutinating titers in cultured yellowtail is shown in Fig. 4. Titers against the KG+ phenotype ranged from 1:8 to 1:128, while against the KG– phenotype they ranged from 1:<4 to 1:4.

DISCUSSION

In a previous study Kitao (1982) reported that antiserum prepared against *Enterococcus seriolicida* passaged several times on an artificial medium (KG+ phenotype) did not agglutinate fresh isolates (KG– phenotype) from diseased fish. Kitao also found that most strains from diseased fish could not be agglutinated with the antiserum raised to the KG+ phenotype. This suggested that the surface composition of *E. seriolicida* was unstable and could be changed easily on artificial culture. In the present study, we demonstrated the relationship between some cell surface properties and virulence of *E. seriolicida*.

Generally, opsonization of bacterial cells is dependent upon binding of complement or specific antibodies. Complement activation and binding on the surface of bacterial cells is considered to be of primary importance in phagocytosis and subsequent killing (Arduino et al. 1994a, b, Harvey et al. 1992). *Streptococcus pneumoniae* (Williams 1988), *Staphylococcus epidermidis* (Johnston et al. 1986), and *Pseudomonas aeruginosa* (Schwarzmann & Boring 1971) possess capsules which inhibit opsonization by complement activation. In the present investigation, no visible capsule surrounding *Enterococcus seriolicida* was observed by light microscopy. However, scanning electron microscopy demonstrated a difference in the cell surface morphology between KG– and KG+ phenotypes. The clumps on the surface of the KG– phenotype may represent capsular material. KG– cells were more resistant to phagocytosis by yellowtail phagocytic cells compared with KG+ cells and the chemiluminescence response to KG– phenotypes was also suppressed. The hydrophilic properties of KG– phenotypes may restrict adhesion to the phagocyte surface, thereby reducing phagocytosis. The respiratory burst, measured by the chemiluminescence response, can usually be induced by the attachment of foreign particles to the surface of phagocytic cells (Welch 1980). Chemiluminescence values of yellowtail phagocytic cells against KG– phenotype did not differ significantly between opsonized and non-opsonized cells, suggesting that the surface material might have relevance to the virulence of KG– phenotypes by inhibiting complement binding to the bacterial surface.

The cell surface hydrophobic properties of pathogenic bacteria are thought to play an important role in host cell attachment. In this study, the KG+ phenotype strain of *Enterococcus seriolicida* showed high hydrophobic properties while the KG– phenotype strain was more hydrophilic. Most pneumococci are encapsulated and bind hexadecane poorly, suggesting that these organisms are not hydrophobic (Irvin 1990). This is similar to the finding with the KG– phenotype strain of *E. seriolicida*. In our laboratory it was found that the cell surface content of hydrophilic monosaccharides such as glucose, rhammnose, and sucrose was higher in the KG– phenotype strains than in the KG+ phenotype strains (data not shown). This finding suggests that KG– phenotypes possess a cell surface capsule that is rich in hydrophilic monosaccharides and that causes this phenotype to be more hydrophilic.

Bacterial surface lectins, which are demonstrable because of their ability to cause agglutination of erythrocytes, are also important factors in host cell attachment (Courtney et al. 1990). However, tests of the hemagglutinating properties of *Enterococcus seriolicida* KG+ or KG– phenotypes revealed no activity in either phenotype. Thus, we could not demonstrate a relationship between hemagglutinating activity of *E. seriolicida* and pathogenicity.

Finally, we investigated the specific immune response of yellowtail to the KG– and KG+ phenotypes. KG+ cells elicited higher serum agglutinating titers than the KG– phenotype. This suggests that the yellowtail has difficulty in recognizing the cell surface of KG– cells as foreign. Kitao (1983) reported that KG– phenotypes showed relatively high virulence compared with KG+ by a challenge test. This finding is in accordance with the results of this study which showed that there was a significantly lower response in phagocytosis, chemiluminescence, and antibody production in yellowtail against the KG– phenotype compared with the KG+ phenotype. Further studies

are in progress to document the presence of the cell surface putative capsule by thin section electron microscopy.

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