Vol. 37: 121–126, 1999 | DISEASES OF AQUATIC ORGANISMS Dis Aquat Org | Published July 30

The protective immune response of yellowtail *Seriola quinqueradiata* to the bacterial fish pathogen *Lactococcus garvieae*

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ABSTRACT: Yellowtail *Seriola quinqueradiata* were immunized with 2 different *Lactococcus garvieae* bacterin, formalin-killed KG– phenotype cells (capsulated phenotype) and formalin-killed KG+ phenotype cells (unencapsulated phenotype). These 2 injected vaccines conferred long-term protection to yellowtail against an artificial infection of an encapsulated *Lactococcus garvieae* strain with long-lasting agglutinating titres against KG+ phenotype cells. However, no agglutinating titres or low agglutinating titres against KG– phenotype cells were detected in fish given each of these bacterin. These results suggested that a capsule in KG– phenotype cells apparently affects their immunogenicity, but the antigens which conferred protection to fish against lactococcal infection may be located on the surface of KG+ phenotype cells, and are not cell capsules in KG– phenotype cells. The protection offered by a formalin-killed KG+ phenotype cell vaccine would not appear to be strain specific. Encapsulated *L. garvieae* cells were well phagocytosed, and fimbrie-like appendages were seen in KG– phenotype cells after treatment with yellowtail immune serum.

KEY WORDS: Vaccines · *Lactococcus garvieae* · *Seriola quinqueradiata* · Phenotypic variation · Immune response

INTRODUCTION

Lactococcal infection in yellowtail *Seriola quinqueradiata* caused by *Lactococcus garvieae* (formerly *Enterococcus seriolicida*) is a serious bacterial disease in Japan (Kitao 1993).

Recently, Eldar et al. (1996) and Teixeira et al. (1996) suggested that *Enterococcus seriolicida* should be reclassified as a synonym of *Lactococcus garvieae* on the basis of DNA-DNA hybridization. *L. garvieae* has been divided into non-agglutinating (KG-) and agglutinating (KG+) phenotype cells using anti-KG+ phenotype serum. The KG– phenotype is agglutinated by anti-KG– serum, but not by antisera to KG+ phenotype cells (KG7409 KG+ phenotype). In contrast, KG+ phenotype strains can be agglutinated with antisera to both KG+ and KG– phenotypes (Kitao 1982, 1993). The KG– factor was found by transmission electron microscopy to be localized in a cell capsule. These capsules inhibited agglutination with anti-KG+ serum (Yoshida et al. 1996a) and was possibly involved in resistance to opsonophagocytosis by yellowtail head kidney phagocytes (Yoshida et al. 1996a, 1997).

The agglutinating titres of KG– phenotype cells with serum obtained from fish immunized with formalin-killed KG– phenotype cells emulsified with Freund's complete adjuvant (FCA) were lower than those against the KG+ phenotype cells. This suggests that the immune system of yellowtail has difficulty in recognizing the cell capsule of the KG– phenotype as a foreign body and this may play an important role in virulence (Alim et al. 1996, Yoshida et al. 1996a, 1997).

There has been an urgent need for protective vaccines against this infection due to the high incidence of lactococcal infection at all stages of yellowtail

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aquaculture (Kitao 1993) and the frequency of multiple-drug resistant strains (Aoki et al. 1990). Recently, a commercial oral vaccine against this infection has become available in Japan. However, no detailed information on cell phenotypes, the protective antigen, duration of protection or agglutination titres against the different phenotype cells was reported. Furthermore, the interaction of both phenotypes of *Lactococcus garvieae* with phagocytic cells and immune serum was poorly defined. Therefore, a better understanding of the pathogenesis of this infection and interactions between the yellowtail host defense system and this pathogen are important.

In previous studies, Yoshida et al. (1996a, 1997) speculated that a cell capsule contributes to the virulence of the KG– phenotype in fish and it is possible that the cell capsules may be the protective antigens in spite of their low immunogenicity in fish. This study was designed to determine whether protection is conferred on fish by intraperitoneal injection with formalin-killed KG– phenotype cells (capsulated), and formalin-killed KG+ phenotype cells (unencapsulated). The duration of effectiveness, agglutinating titres, and the interactions of *Lactococcus garvieae* with phagocytic cells and immune serum were also measured.

MATERIALS AND METHODS

Bacteria. *Lactococcus garvieae* MS93003 KG– and KG+ phenotype, and KG9502 KG– phenotype were used in this study. Bacterial properties are shown in Table 1 in the 'Results'. MS93003 KG+ and NG8206 KG+ phenotype cells were obtained after subculturing on Todd-Hewitt agar (THA; Difco, Detroit, Michigan, USA) containing 2,3,5-triphenyltetrazolium chloride (TTC) (Kitao 1982). Cell capsulation was determined by antiserum against KG– and KG+ phenotype cells (Yoshida et al. 1996a) and transmission electron microscopy (TEM).

Antiserum against phenotypes. Antisera against both phenotype cells were raised as described by Yoshida et al. (1996a). Rabbit antisera with a titre over of 1:1280 (agglutinating titres) against the homologous phenotype cells were used. Antisera were heated at 56°C for 30 min and then kept at –80°C until required.

Confirmation of cell capsule by TEM. *Lactococcus garvieae* KG– and KG+ phenotype cells were grown overnight in 10 ml of Todd-Hewitt broth (THB). The bacteria were suspended in 0.3% formaldehyde solution and held overnight at 4°C. They were washed 3 times with phosphate-buffered saline (PBS) and resuspended in 10 ml of a 1:320 dilution of KG– antiserum in PBS which had previously been adsorbed with cells of the poorly encapsulated strain (NG8206, KG+ phenotype cells; Yoshida et al. 1996a, 1997) to remove antibodies directed against the KG+ factor. Bacterial cells were embedded and cut into thin sections as described by Yoshida et al. (1997).

Bacterin preparation. Cultures of each phenotype of MS93003 in THB were harvested and killed by adding formaldehyde to a final concentration of 0.3% at 4°C for 24 h. The bacteria were washed twice with physiological saline and adjusted to an optical density of 1.0 at 620 nm.

Fish. Yellowtail used in this study were bred by the Miyazaki Experimental Fisheries Station, Miyazaki, Japan, and kept in a net cage near the Fisheries station. Before the experiment, fish (n = 10) were subjected to a bacteriological examination to determine the presence of *Lactococcus garvieae*. The fish mean weight at immunization was 105 g and was approximately 119 g at 14 d, 284 g at 65 d, 660 g at 135 d, 978 g at 295 d, and 1.4 kg at 358 d after immunization.

Vaccination. The fish were immunized by intraperitoneal injection of 0.5 ml of formalin-killed KG– phenotype cells (FKC-KG–) and formalin-killed KG+ phenotype cells (FKC-KG+). Control fish were injected with 0.5 ml of 0.85% saline. At 14, 65, 135, and 295 d after immunization, fish were challenged with the MS93003 KG– phenotype (5.2×10^5 cfu fish^{-1} at 14, 3.8×10^5 cfu fish^{-1} at 65, 2.6×10^5 cfu fish^{-1} at 135, and 4.6×10^5 cfu fish^{-1} at 295 d, respectively) by intraperitoneal injection. Fish were monitored for 14 d after infection and all dead fish subjected to bacterial examination. After 14 d, all surviving fish were sacrificed and similarly examined. At 358 d after immunization, fish immunized with FKC-KG+ were challenged with KG9502 KG– phenotype cells (2.1×10^5 cfu fish^{-1}).

Agglutinating titres in immunized fish. Blood samples were obtained from 5 immunized and 5 control fish. Serum was obtained from blood after clotting at room temperature and centrifugation at $1000 \times g$ for 10 min. The agglutinating titres of the serum samples against both phenotypes of MS93003 were determined according to Roberson (1990).

Passive immunization against artificial infection. At 135 d after immunization, serum from fish (n = 5) immunized with FKC-KG+ phenotype bacterin was obtained, mixed and sterilized by filtration using a 0.45 µm filter. Agglutinating titres of immune serum against KG– and KG+ phenotype cells were 1:4 and 1:64, respectively. Immune sera were kept at –80°C until required for use. Fish with a mean body weight of 45 g (n = 13, 10 for infection test, 3 for TEM samples) were passively immunized intraperitoneally with 3 ml of immune serum. Twenty hours after administration of serum, fish were challenged by intraperitoneal injection with *Lactococcus garvieae* KG9502 KG– pheno-

type cells at a density of 2.5×10^4 cfu $fish^{-1}$. Controls were given 3 ml physiological saline. After 20 h infection, kidney and spleen in infected fish (n = 3) were sampled for TEM to examine the *in vitro* morphology.

Attachment or injestion of bacteria by head kidney cells. Equal volumes of KG- or KG+ phenotype cells (1.0×10^6 cfu ml^{-1}) and yellowtail normal pooled sera (n = 5) were mixed and incubated at 15°C for 1 h. Serum from fish immunized with FKC-KG+ and FKC-KG- (at 135 d after immunization) were mixed and opsonized with an equal volume of KG- phenotype cells (1.0×10^6 cfu ml^{-1}) at 15°C for 1 h. The bacteria did not agglutinate on this occasion. Opsonized cells then were washed 3 times with Hanks' balanced salt solution (HBSS), and adjusted to an optical density of 0.6 at 620 nm in HBSS. Head kidney phagocytic cells were obtained separately from yellowtail (mean body weight 1250 g, n = 3) using the method of Braun-Nesje et al. (1982), then mixed and adjusted to 6.0×10^6 cells ml^{-1} in HBSS and allowed to adhere to cover glass for 2 h. A phagocytosis test was performed as described by Yoshida et al. (1996a).

Statistical analysis. Statistical analyses of the protective efficacy of the vaccines were performed by Fisher's protected least-squares difference. Significance between agglutinating titres of immunized fish and control fish are analysed using Student's *t*-test. Phagocytosis assay was analysed using Duncan's multiple range test.

Table 1. *Lactococcus garvieae*. Bacterial strains used in this study and their properties

Strain	Year	Antiserum KG-	Antiserum KG+	Capsulation (size) by TEM	Source (prefecture)
MS93003 (KG-)	1993	+	-	+ (20-40 nm)	Miyazaki
MS93003 (KG+)[a]	1993	+	+	-	Miyazaki
KG9502 (KG-)	1995	+	-	+ (40-100 nm)	Kagoshima
NG8206 (KG+)	1982	+	+	-	Nagasaki

[a]MS93003 KG+ phenotype cells were obtained after subculutring MS93003 KG- phenotype cells on Todd Hewitt agar containing 2,3,5-triphenyltetrazolium chrolide (TTC)

RESULTS

Bacterial strain for bacterin

KG- phenotype cells incubated with rabbit KG- antiserum and stained with ruthenium red demonstrated a capsular layer ranging from 20 to 40 nm in MS93003, and 40 to 100 nm in KG9502 KG- phenotype cells. No layer was detected in MS93003 KG+ phenotype (Table 1).

Efficacy of vaccines

The efficacy of vaccines against artificial infection is shown in Table 2. Efficacy of these vaccines (formalin-killed KG- phenotype; FKC-KG-, and formalin-killed KG+ phenotype; FKC-KG+) was observed until 294 d after immunization. After challenge with *Lactococcus garvieae* MS93003 there were no mortalities among fish immunized with FKC-KG+; 2 died at 64 d among the KG- phenotype vaccinates. No mortalities occurred in any of the vaccinates and control fish before artificial infection with *L. garvieae*. No bacteria were isolated from survivors in vaccinated fish 14 d after infection. At 358 d after immunization with the FKC-KG+ phenotype, fish were infected with *L. garvieae* KG9502 KG- phenotype cells. Protection was demonstrated also against this strain. However, *L. garavieae* was recovered from 2 surviving fish 14 d after infection.

Agglutinating titres

Agglutinating titres against KG+ phenotype cells in sera obtained from immunized fish are shown in Fig. 1. High titres against the KG+ phenotype cells in the fish immunized with this phenotype were detected throughout the experiments, but not against the KG- phenotype (<1:4 to 1:4). Agglutinating titres against KG+ phenotype cells in FKC-KG- cell-immunized fish were significantly higher 14 to 134 d after immunization but not after 296 d. Agglutinating titres against KG- phenotype cells in fish immunized with FKC-KG- were <1:4 to 1:4 throughout the experiments.

Table 2. *Seriola quinqueradiata* infected with *Lactococcus garvieae*. Fish mortality (numbers of dead fish/numbers of infected fish) during immunization with formlain killed MS93003 KG- and KG+ phenotype cells. Results were significantly different from those of non-treated fish ($^{*}p < 0.05$, $^{**}p < 0.01$)

Vaccine	Days after immunization 14	65	135	295	358
Formalin-killed					
KG- phenotype	0/10**	2/10*	0/10**	0/10**	
KG+ phenotype	0/10**	0/10**	0/10**	0/10**	1/8[a]
Control					
Non-treated	9/10	8/10	8/10	8/10	7/8[a]

[a]Fish were infected with *Lactococcus garvieae* KG9502 KG- phenotype cells

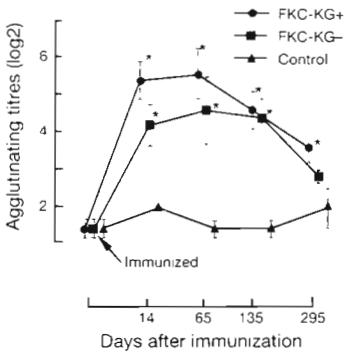

Fig. 1. *Lactococcus garvieae* infecting *Seriola quinqueradiata*. Agglutinating titres against KG+ phenotype cells in serum from fish immunized with MS93003 FKC-KG– phenotype cells, FKC-KG+ phenotype cells or non-treated fish. Agglutinating titres against KG– phenotype cells in fish immunized with vaccines were <1:4 to 1:4 throughout the experiment. *Results were significantly different from those of control fish ($p < 0.05$)

Passive immunization

No mortalities were observed in fish passively given immune serum (n = 10), while 60% of fish given physiological saline died after challenge.

Phagocytosis

The phagocytic rate was higher with KG+ than with KG– phenotype cells opsonized with normal yellowtail serum. Phagocytic activity was enhanced when KG– phenotype cells were opsonized with immune serum compared with normal serum (Table 3).

In vivo and *in vitro* morphology of *Lactococcus garvieae*

Lactococcus garvieae ingested *in vitro* by yellowtail phagocytic cells were seen to be enclosed in phagosomes with some degree of destruction evident after 3 h. (Fig. 2a). In passively immunized fish, KG– phenotype cells with fimbriae-like appendages were phagocytosed and destroyed in the vacuoles (Fig. 2b). In KG– phenotype cells incubated with yellowtail immune serum, appendages were seen projecting from bacterial cells and the cell capsules were incomplete (Fig. 2c,d).

Table 3. Phagocytic response of yellowtail *Seriola quinqueradiata* phagocytic cells against the KG– and KG+ phenotypes of *Lactococcus garvieae*. Values that do not share a common letter are significantly different ($p < 0.05$)

Strain (phenotype)	Phagocytic rate (%)
Opsonized with normal serum	
MS93003 (KG+)	23.3 ± 2.7a
MS93003 (KG–)	11.0 ± 2.3b
KG9502 (KG–)	7.3 ± 1.5b
Opsonized with immune serum[a]	
MS93003 (KG–)	36.7 ± 2.9c
KG9502 (KG–)	25.7 ± 3.5a

[a]Agglutinating titers of immune serum against KG– and KG+ phenotype cells were 1:4 and 1:64, respectively

DISCUSSION

The antigenic conversion of *Lactococcus garvieae* occurred after several subcultures on KF *Streptococcus* agar supplemented with TTC. KG– phenotype (non-agglutinating strain against KG+ antiserum) strains were more virulent than KG+ cells (Kitao 1983, Alim et al. 1996).

A common strategy for bacteria to avoid the host defense system is the production of anti-phagocytic surface components. Encapsulated bacterial pathogens resist phagocytosis because of reduced binding of serum opsonins and inaccessibility of ligands required for phagocyte binding (Czuprynski 1988). *Streptococcus pneumoniae* and other Gram-positive pathogens produce capsules which contribute to virulence because of their resistance to phagocytosis (Williams 1988). In a β-haemolytic *Streptococcus* spp. pathogenic for rainbow trout, the capsule plays a role in resistance to opsonophagocytosis by trout macrophages and leads to mortalities in fish (Yoshida et al. 1996b). Yoshida et al. (1996a) reported similar findings for the *Lactococcus garvieae* KG– phenotype (encapsulated), which was more hydrophilic than the KG+ variant (unencapsulated) and resistant to phagocytosis by yellowtail head kidney phagocytes. Furthermore, in KG– cells incubated with anti KG– phenotype serum and stained with ruthenuium red, various-sized capsules were seen adjacent to the cell wall. These were thought to play a role in resistance to opsonophagocytosis and to affect immunogenicity in yellowtail (Yoshida et al. 1997).

Preliminary investigations (Iida et al. 1982, Sato et al. 1996) have shown protection with increasing opsonic activities of fish immunized with *Lactococcus garvieae* formalin-killed cells. However, no detailed information on the antigenicity of *L. garvieae* phenotypes and the duration of protection was given. In the present study, antigens conferring protection to yellowtail and the immune response were analyzed using formalin-killed KG+ and KG– phenotype cell vaccines.

Yoshida et al. (1996a) reported that low agglutinating titres against KG– phenotype cells were detected on immunization of KG– emulsified with adjuvant. However, the agglutinating titres of KG– phenotype

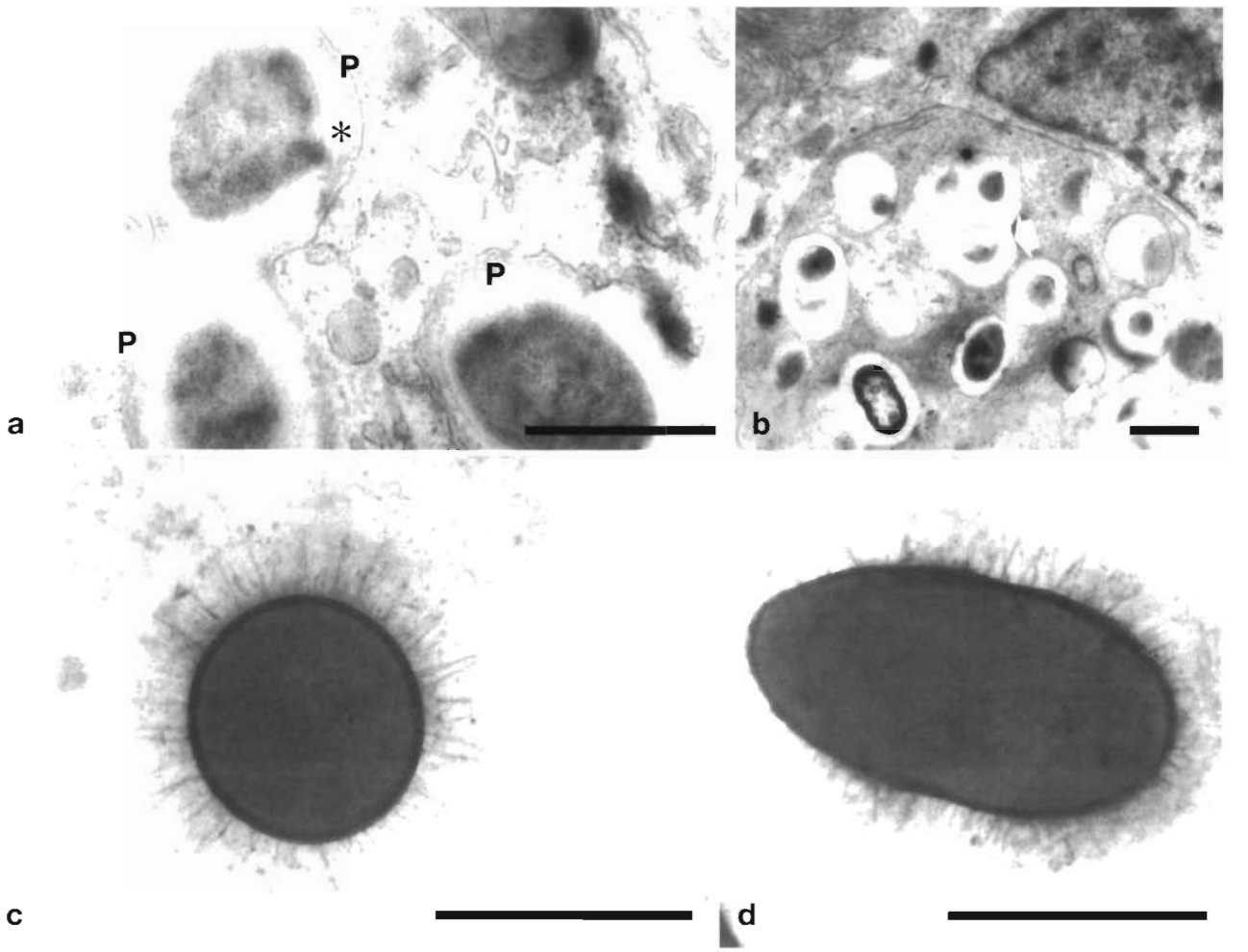

Fig. 2. *Lactococcus garvieae* infecting *Seriola quinqueradiata*. (a) At 3 h post phagocytosis assay, *L. garvieae* is found in phagosome (P) with a partial bacterial lysis (*). (b) At 1 d post infection with *L. garvieae*, fish transplanted with immune serum showed bacterial cells in vacuoles with bacterial destruction (arrows). (c,d) *L. garvieae* incubated with immune serum showing fimbrie-like structures with a partial deficiency of cell capsule. Scale bars = 1 µm

cells were very low in farmed fish compared to titers against the KG+ phenotype. This study supported these findings, with low agglutinating titres (<1:4 to 1:4) against KG– phenotype cells being detected in fish immunized with KG– cells. The cell capsule of *Lactococcus garvieae* is thought to be responsible for the low immunogenicity in yellowtail. However, formalin-killed KG– and KG+ phenotype bacterin elicited agglutinating titres against KG+ phenotype cells. These results may suggest that the capsule in KG– phenotype cells covered the agglutinating site against the KG+ agglutinating antibody. The effectiveness of each bacterin had a duration of at least 10 mo as shown by agglutinating titres against the KG+ phenotype. Both bacterins were effective against infection with the KG– phenotype cells, suggesting that the protective antigen against *L. garvieae* infection is located on the surface of KG+ phenotype cells or projects into capsules from the surface of KG+. Further investigations on the surface morphology of KG+ phenotype cells are in progress.

Appendages were seen extending from the cell surface of *Lactococcus garvieae* with some disruption of the cell capsule after opsonization with yellowtail immune serum. This finding may indicate that immune serum with unknown factors as complements affects the stability of the cell capsule in KG– phenotype cells. These changes were not evident after treatment with rabbit immune serum which previously had been adsorbed with cells of poorly encapsulated strains. Furthermore, most of *L. garvieae* cells with appendages were destroyed in intracellular vacuoles after ingestion by fish phagocytes. Low agglutinating titres against KG– phenotype cells (1:4) were even detected in the

serum obtained from fish immunized with the FKC-KG+ phenotype, and the rate of phagocytosis was enhanced after immune serum opsonization as compared to the normal serum opsonization. These results may suggest that immune serum with low agglutinating titres against the KG– phenotype promote phagocytosis and play an important role in the defense mechanisms against lactococcal infection in vaccinated fish. Harvey et al. (1992) and Arduino et al. (1994a,b) reported that complement was of primary importance in the killing of enterococci in human polymorphonuclear phagocytic cells. They also found that a small amount of specific antibodies promoted greater killing by phagocytes than that which occurred in the presence of an active complement alone. Further investigations are in progress to clarify the nature, function and antigenicity of appendages of KG– phenotype cells, and the stabilities of cell capsules in the presence of a complement.

Acknowledgements. This study was supported by a grant-in-aid for scientific research from the Ministry of Education, Science and Culture of Japan.

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Editorial responsibility: David Bruno, Aberdeen, Scotland, UK

Submitted: June 3, 1998; Accepted: April 12, 1999
Proofs received from author(s): June 25, 1999