The protective immune response to the bacterial fish pathogen, *Lactococcus garvieae* and application of its attenuated live *L. garvieae* cells in yellowtail, *Seriola quinqueradiata*

（魚病細菌 *Lactococcus garvieae* に対するブリの免疫防御反応と弱毒生ワクチンのブリへの応用に関する研究）

Tsuyoshi Ooyama

2007

Table of contents

List of Tables

Pages

List of Figures

Pages

Fig.4. *Lactococcus garvieae.* Detection of proteins extracted from KG9408, MS93003 and NSS9310 strains by immunoblotting.(A)Proteins on filters reacted with rabbit anti-KG9408 serum (Panel A) and rabbit anti-MS93003 (Panel B); Lane M: Marker proteins;Lanes 1 and 8: NSS9310; Lanes 2 and 7: MS93003; Lane 3 and 6: KG9408; Lanes 4 and 5: *L. lactis* ATCC19435; 2 protein bands of approximately 15 to 20and 30 to 35kDa can be seen in KG9408 and MS93003, but not in NSS9310 (arrowheads). (B) Proteins on filters reacted with yellowtail anti-KG9408 serum (Panel A) and yellowtail anti-MS93003 serum (Panel B); Lane M: marker proteins;Lanes 1 and 8: *L.lactis* ATCC19435; Lanes 2 and 7: KG9408; Lanes 3 and 6: MS93003; Lanes 4 and 5: NSS9310; increase in staining intensity is seen for proteins of approx. 30 to 35 and 45 to 50 kDa in KG9408 and MS93003 compared to NSS9310 (arrowheads). (p.65)

Fig.5. Virulent (A) and attenuated (B) cells of *Lactococcus garvieae* cultured in filtered yellowtail serum. A: MS93003V (virulent) shows fimbriae (indicated by an arrow) with a well-developed capsule on its cell surface. B: MS93003A (attenuated) shows peritrichous short fimbriae (indicated by an arrow) without a well-developed capsule on its cell surface. Bars indicate 1 μm. (p.81)

Abstract

Yellowtail, *Seriola quinqueradiata* were immunized with two different *Lactococcus garvieae* bacterins, formalin-killed KG − phenotype cells (capsulated phenotype) and formalin-killed KG+ phenotype cells (non-capsulated phenotype). Inoculation of either of these two vaccines conferred long-term protection to yellowtail against challenge with the capsulated *Lactococcus garvieae* strain. High agglutinating titres against KG+ phenotype cells persisted for 135 days but no or only low agglutinating titres against KG − phenotype cells were detected in fish given either bacterin. These results suggest that a capsule on KG − phenotype cells affects its immunogenicity, but the antigens conferring protection against lactococcal infection to fish may be located on the surface of KG+ phenotype cells and not in the cell capsule. The protection offered by formalin-killed KG+ phenotype cell vaccine was not strain specific. Capsulated *L. garvieae* cells were phagocytosed well and fimbriae-like appendages were seen in KG − phenotype cells after treatment with immune serum.

The cell surface components of strains of *Lactococcus garvieae* were examined. Two capsular types of *L. garvieae* were found; one with a highly developed capsule (KG9408) and one with a micro-capsule (MS93003) carrying fimbriae-like components projecting from cell surface. One strain (NSS9310) had neither cell capsule nor fimbriae-like structures on its cell surface. The strains with the highly developed capsule were more virulent to fish than either the micro-capsular or non-capsular. The KG9408, MS93003

and NSS9310 strains could be clearly differentiated by their susceptibility to bacteriophages. Protection against *L. garvieae* infection was induced in yellowtail, *Seriola quinqueradiata* by immunization with formalin-killed *L. garvieae* KG9408 and MS93003 cells. Although protection was also induced by immunization with NSS9310, the level of protection was significantly lower than that with KG9408 and MS93003 vaccines. Passive immunization with yellowtail immune sera raised against KG9408 and MS93003 conferred strong protection to yellowtail with rapid bacterial clearance after challenge with *L. garvieae*. Immunoblotting analysis of protein antigens extracted from *L. garvieae* strains using rabbit anti-KG9408 and anti-MS93003 sera, and yellowtail anti-KG9408 and anti-MS93003 sera indicated that some bands seen in KG9408 and MS93003 strains were not detectable in NSS9310.

An attenuated *Lactococcus garvieae* strain lacking a virulence-associated capsule on its cell surface was evaluated for its application as a live vaccine. The attenuated strain (MS93003A) was obtained from the parent strain (MS93003V), which produced a well-developed capsule, by culturing on an agar medium supplemented with 2,3,5-triphenyltetrazolium chloride (TTC). When live cells of *L. garvieae* (MS93003A) or formalin-killed cells (MS93003A) were used as an injectable vaccine, protection against virulent *L. garvieae* (MS93003V) was conferred on *Seriola quiqueradiata*. Furthermore, at a relatively lower water temperature and using a lower dose of the cells, application of live cells of *L. garvieae* (MS93003A) conferred a stronger immunity to fish when compared with that conferred by

formalin-killed cells (MS93003A).

The MS93003A cells did not recover their virulence even after *in vivo* passages in fish. MS93003A live cells also conferred long-lasting protective immunity to *S. quinqueradiata* against virulent *L. garvieae* infection.

日本語要旨

ブリを魚病細菌 Lactococcus garvieae（レンサ球菌）の莢膜保有株（MS93003；ＫＧ－型）および莢膜欠失株（MS93003；ＫＧ＋型）のホルマリン不活化菌体で免疫した。両ホルマリン不活化菌体共に，ブリに長期の感染防御を誘導した。ブリの血清中には，ＫＧ＋株に対しては高い凝集抗体価が認められたが，ＫＧ－株には弱い凝集抗体価か全く凝集を示さなかった。この結果，ＫＧ－株の莢膜は，ブリに対して免疫原性は低く，しかも防御抗原ではないと考えられた。さらに防御抗原は，莢膜ではなく，ＫＧ＋株の細胞表面に存在していることが示唆された。ホルマリン不活化ＫＧ＋菌体で免疫したブリの感染防御には，株特異性は認められなかった。莢膜を有する菌株であるＫＧ－株は，ワクチンしたブリの血清と反応させることで，ブリの貪食細胞に十分に貪食されることが判明した。また，ＫＧ－株において，線毛様構造物が観察された。

レンサ球菌株の細胞表面構造について研究した。その結果，大きな莢膜を有する菌株（KG9408）と微細莢膜を有する菌株（MS93003）が認められた。両細菌にも，線毛様構造物が観察された。また，莢膜および線毛様構造物ともにまったく観察されない菌株（NSS9310）も認められた。大きな莢膜を有する菌株（KG9408）のブリに対する毒性は，微細莢膜を有する菌株（MS93003）又は微細莢膜および莢膜も有しない菌株（NSS9310）よりも強かった。バクテリオファージに対する感受性は，これら３株で明らかな違いが認められた。さらに，KG9408 株および MS93003 株のホルマリン不活化菌体は，ブリに防御免疫を誘導した。しかし，NSS9310 株の不活化菌体の免疫による防御免疫は，KG9408 株および MS93003 株の免疫よりも顕著に低かった。ぞれぞれのホルマリン不活化菌体で免疫したブリの血清を分離し，健康なブリにその血清を移入した。その結果，KG9408 株および MS93003 株で免疫したブリより分離した血清を移入した個体で，強い感染防御が観察された。これらブリの免疫血清を用いて，それぞれの菌体の蛋白質を，免疫染色を施し解析した。その結果，KG9408 株および MS93003 株のみに認められるが，NSS9310

株には発現していない蛋白のバンドが検出された。

莢膜を有する毒性の強いMS93003V株を，TTC(2,3,5ートリフェニルテトラゾリウムクロライド)を含有した寒天培地で継代培養することにより，元株のMS93003V株からMS93003A株を分離した。MS93003A株は，莢膜は欠失しているが，線毛を細胞表面に保有している菌株であった。MS93003A株は，ブリに対して病原性がないが，そのホルマリン不活化菌体のブリへの免疫は，感染防御を強く誘導した。そのため，病原性に関わる莢膜を欠失させた菌株であるMS93003A株を弱毒生ワクチンに応用した。その結果，MS93003A株の生ワクチンは，ホルマリン不活化菌体より免疫細菌数が低くても，また，低温下（１７℃）で免疫しても十分な感染防御をブリに誘導することが確認できた。さらに，生体内通過を行った後でも病原性の回復は認められなかった。

Acknowledgements

I would like to express my warm appreciation to Drs. Toshiaki Itami , Masahiro Sakai, and Terutoyo Yoshida, University of Miyazaki, and Jun Yamamoto, University of Kagoshima for their invaluable guidance and inspiration to the research leading to this dissertation.

I also would like to express my sincere thanks to Dr. V. Inglis for reviewing parts of this thesis, and to Dr. J. Carson, Fish Health Unit, Department of Primary Industries, Water and Environment, Tasmania for providing *L. garviae* strains from rainbow trout.

Further my sincere thanks should be expressed to members of the Miyazaki prefecture; fisheries experimental station for their help to complete this thesis.

Chapter I

General introduction to *Lactococcus garvieae* infection in marine aquaculture in Japan

Lactococcus garvieae (formerly referred to as *Enterococcus seriolicida*) infection has been causing a serious bacterial fish disease in Japan (Kusuda *et al.*, 1991). This organism was isolated from not only farmed fish such as the yellowtail *Seriola quinqueradiata* and the amberjack *S. dumerili* (Ooyama *et al.*, 2002) but also the rainbow trout *Oncorhynchus mykiss* (Carson *et al.*, 2002; Eldar and Ghittino, 1999), the giant prawn *Macrobrachium rosenbergii* (Chen *et al.*, 2001), and mammals (Kawakami *et al.*, 2005 and 2006). *L. garvieae* infection is currently one of the most serious bacterial fish diseases in aquaculture industries all over the world.

L. garvieae strains isolated from the diseased fish in Japan were divided into two phenotypes—non-agglutinating (capsulated strain: KG－) and agglutinating (non-capsulated strain: KG+)—by using a rabbit antiserum raised against cells with the non-capsulated phenotype (Kitao, 1982; Alim *et al.*, 1996; Yoshida *et al.*, 1997). As compared to the non-capsulated strain, the capsulated strain is more virulent to the yellowtail because the latter strain is resistant to the opsonophagocytosis of the yellowtail phagocytic cells (Yoshida *et al.*, 1996 and 1997). Kawanishi *et al.* (2006) reported that *L. garvieae* strains isolated from the diseased fish of the species *S. quinqueradiata*, *S. dumerili*, and *S. lalandi* farmed in Japan were virulent to the yellowtail, while those isolated from the rainbow trout and other sources

were less virulent or non-virulent to it. Moreover, bacteriophage typing differentiated the *L. garvieae* strains isolated from the three above-mentioned *Seriola* species from those isolated from other sources. Furthermore, the chromosomal DNA restriction patterns of *L. garvieae* obtained by pulsed-field gel electrophoresis (PFGE) analysis revealed a large heterogeneity among the *L. garvieae* strains isolated from different sources. These results indicated that the *L. garvieae* strains isolated from the farmed fish in Japan were different from those isolated from other sources, and strains isolated from mammals and the rainbow trout caused less damage or no damage to marine aquaculture industries in Japan.

Oral and injectable vaccines have been developed and applied to aquaculture (Kawanishi *et al.*, 2005); since then, the economic damage caused by *L. garvieae* infection has decreased drastically in Japan (Fukuda, 1999). Although the efficacy of these vaccines in preventing *L. garvieae* infection has been demonstrated in Japan (Nakanishi, 2003), the precise mechanism of this protective immunity remains unknown. Ooyama *et al.* (1999) reported that a formalin-killed *L. garvieae* injectable vaccine induced strong protective immunity in the yellowtail, and the immunity lasted for at least 1 year.

In this Ph.D. thesis, the *L. garvieae* antigens associated with protective immunity in the yellowtail and amberjack, the protective duration of vaccines, and the virulence of *L. garvieae* were investigated. Moreover, an attenuated *L. garvieae* strain was created from the virulent strain by excluding the virulence-associated capsule on its cell surface, and the

attenuated strain was applied as a live vaccine. This thesis includes the following chapters: "General introduction to *Lactococcus garvieae* infection in marine aquaculture in Japan" (Chapter I), "Discussion and conclusion" (Chapter V), and three chapters that are published manuscripts—"The protective immune response of yellowtail *Seriola quinqueradiata* to the bacterial fish pathogen *Lactococcus garvieae*" (Chapter II), "Cell-surface properties of *Lactococcus garvieae* strains and their immunogenicity in the yellowtail *Seriola quinqueradiata*" (Chapter III), and "Application of attenuated *Lactococcus garvieae* strain lacking a virulence-associated capsule on its cell surface as a live vaccine in yellowtail *Seriola quinqueradiata* Temminck and Schlegel" (Chapter IV). Chapters II and III have been published in "DISEASES OF AQUATIC ORGANISMS" (Ooyama *et al.*, 1999; Ooyama *et al.*, 2002) and Chapter IV, "Journal of Applied Ichthyology" (Ooyama *et al.*, 2006).

References

Alim SR, Kawai K, and Kusuda R (1996): Comparative pathogenicity study on antigenically variant strains of *Enterococcus seriolicida*. J. Fish Dis., 19:39-46

Carson J, Gudkovs N, and Austin B (1993): Characteristics of an *Enterococcus*-like bacterium from Australia and South Africa, pathogenic for rainbow trout, *Oncorhynchus mykiss* (Walbaum). J. Fish Dis., 16:381-388

Chen SC, Lin YD, Liaw LL, and Wang PC (2001): *Lactococcus garvieae* infection in the giant freshwater prawn *Macrobrachium rosenbergii* confirmed by polymerase chain reaction and 16S rDNA sequencing. Dis. Aquat. Org., 45:45-52

Eldar A, Ghittino C, Asanta L, Bozzetta E, Goria M, Prearo M, and Bercovier H (1996): *Enterococcus seriolicida* is a junior synonym of *Lactococcus garvieae*, a causative agent of septicemia and meningoencephalitis in fish. Curr. Microbiol., 32:85-88

Fukuda Y (1999): Diseases of marine fishes and shellfishes cultured in Oita Prefecture diagnosed from 1980 to 1997. Bull. Oita Inst. Mar. Fish. Sci., 2:41-74 (written in Japanese)

Kawanishi M, Kojima A, Ishihara K, Esaki H, Kijima M, Takahashi T, Suzuki S, and Tamura T (2005): Drug resistance and pulsed-field gel electrophoresis patterns of *Lactococcus garvieae* isolates from cultured *Seriola* (yellowtail, amberjack and kingfish) in Japan. Lett. Appl. Microbiol., 40:322-328

Kawanishi M, Yoshida T, Yagashiro S, Kijima M, Yagyu K, Nakai T, Murakami M, Morita H, and Suzuki S (2006): Differences between *Lactococcus garvieae* isolated from the genus *Seriola* in Japan and those isolated from other animals (trout, terrestrial animals from Europe) with regard to pathogenicity, phage susceptibility, and genetic characterization. J. Appl. Microbiol., 101:496-504

Kusuda R, Kawai K, Salati F, Banner CR, and Fryer JL (1991): *Enterococcus seriolicida* sp. nov., a fish pathogen. Int. J. Syst. Bacteriol., 41:406-409

Nakanishi T (2003): Current status of the use and development of fish vaccines in Japan and overseas. Proc. Japan Fish. Resour. Conserv. Assoc., 9:7-12 (written in Japanese)

Ooyama T, Kera A, Okada T, Inglis V, and Yoshida T (1999): The protective immune response of yellowtail *Seriola quinqueradiata* to the bacterial fish pathogen *Lactococcus garvieae*. Dis. Aquat. Org., 37:121-126

Ooyama T, Hirokawa Y, Minami T, Yasuda H, Nakai T, Endo M, Ruangpan L, and Yoshida T (2002): Cell-surface properties of *Lactococcus garvieae* strains and their immunogenicity in the yellowtail *Seriola quinqueradiata*. Dis. Aquat. Org., 51:169-177

Ooyama T, Shimahara Y, Nomoto R, Yasuda H, Iwata K, Nakamura A, Itami T, and Yoshida T (2006): Application of attenuated *Lactococcus garvieae* strain lacking a virulence-associated capsule on its cell surface as a live vaccine in yellowtail *Seriola quinqueradiata* Temminck and Schlegel. J. Appl. Ichthyol., 22:149-152

Yoshida T, Eshima T, Wada Y, Yamada Y, Kakizaki E, Sakai M, Kitao T, and

Inglis V (1996): Phenotypic variation associated with anti-phagocytic factor in the bacterial fish pathogen *Enterococcus seriolicida*. Dis. Aquat. Org., 25:81-86

Yoshida T, Endo M, Sakai M, and Inglis V (1997): A cell capsule with possible involvement in resistance to opsonophagocytosis in *Enterococcus seriolicida* from yellowtail *Seriola quinqueradiata*. Dis. Aquat. Org., 29:233-235

Chapter II

The protective immune response of yellowtail, *Seriola quniqueradiata* to bacterial fish pathogen *Lactococcus garvieae*

Introduction

Lactococcal infection in yellowtail *Seriola quinqueradiata* caused by *Lactococcus garvieae* (formally *Enterococcus seriolicida*) is a serious bacterial disease in Japan (Kitao, 1993; Kusuda *et al.*, 1991).

Recently, Eldar *et al.* (1996) and Teixeira *et al.* (1996) suggested that *Enterococcus seriolicida* should be re-classified as a synonym of *L. 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. 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 and 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 a possibly involved in resistance to opsonophagocytosis by yellowtail head kidney phagocytes (Yoshida *et al.* 1996a and 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 immuno system of yellowtail has difficulty in recognizing the cell capsule of the KG− phenotype as foreign body and this may play an important role in virulence (Alim *et al.*, 1996, Yoshida *et al.*, 1996a and 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 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 *L. 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 and 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 (non-capsulated). The duration of effectiveness, agglutinating titres, and the interactions of *L. 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 'Result'. MS93003 KG+ and NG8206 KG+ phenotype cells were obtained after subculturing on Todd-Hewitt agar (THA; Difco, Detoroit, 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℃ for 30 min and then kept at −80℃ until required.

Confirmation of cell capsule by transmission electron microscopy (TEM).

L. garvieae KG− and KG+ phenotype cells were grown overnight in 10ml of Todd-Hewitt broth (THB). The bacteria were suspended in 0.3% formaldehyde solution and held overnight at 4℃. They were washed 3 times with phosphate-buffered saline (PBS) and resuspended in 10ml of a 1:320

dilution of KG− antiserum in PBS which had previously been adsorbed with cells of the poorly capsulated strain (NG8206, KG+ phenotype cells; Yoshida *et al.*, 1996a and 1997) to remove antibodies directed against the KG+ factor. After 2h at 4℃, bacteria were washed three times with 0.85% saline and capsules were stained with 0.15% ruthenium red (Sigma, USA). Bacterial cells (then) were embedded in Quetol 651 (Nishin EM, Tokyo, Japan). Thin sections (30nm) were postfixed with uranyl acetate and lead acetate and observed by transmission electron microscopy (Hitachi-H4800MU, Japan) at an accelerating voltage of 100KV.

Bacterin preparation.

Cultures of each phenotype of MS93003 in Todd Hewitt broth (THB) were harvested and killed by adding formaldehyde to a final concentration of 0.3% at 4℃ for 24h. The bacteria were washed twice with physiological saline , and adjusted to an optical density of 1.0 at 620nm.

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 *L. garvieae*. The fish mean weight at immunization was 105g and was approximately 119g at 15 days, 284g at 65 days, 660g at 135 days, 978g at 295 days, and 1.4 Kg at 358 days 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 fifteen, 65, 135, and 295 days after immunization, fish were challenged with the MS93003 KG− phenotype (5.2×10^5 CFU $fish^{-1}$ at 15, 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 days, respectively) by intraperitoneal injection. Fish were monitored for 14 days after infection and all dead fish subjected to bacterial examination. After 14 days, all surviving fish were sacrified and similarly examined. At 358 days after immunization, fish immunized with FKC-KG+ were challenged with MZ9502 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 1,000×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 days after immunization, serum from fish(n=5) immunized with FKC-KG+ phenotype bacterin was obtained, mixed and sterilized by filtration using a 0.45 μ m 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℃ until required for use. Fish with a mean body weight of 45g (n=13, 10 for injection test, 3 for TEM samples) were passively immunized intraperitoneally with 3 ml of immuno serum. Twenty hours after administration of serum, fish were challenged by intraperitoneal injection with *L. garvieae* KG9502 KG− phenotype cells at a density of 2.5×10^4 CFU $fish^{-1}$. Controls were given 3ml of physiological saline. After 20 h infection, kidney and spleen in infected fish (n=3) were sampled for TEM to examine the morphology.

Attachment or ingestion of bacteria by head kidney cells (phagocytosis).

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℃ for 1h. Serum from fish immunized with FKC-KG+ and FKC-KG−(at 135 days after immunaization) were mixed and opsonized with an equal volume of KG − phenotype cells (1.0×10^6 CFU ml^{-1}) at 15℃ for 1h. 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 620nm in HBSS. Head kidney phagocytic cells were obtained separately from yellowtail (mean body weight 1250g, n=3) using the method of Braun-Nesje *et al.* (1982), then mixed and adjusted to 6.0×10^6 CFU ml^{-1} in HBSS and allowed to adhere to coverglass for 2h. Previously opsonized *L. garvieae* cells (10^5) were overlayed to coverglass and incubated at 25℃ for 1h, rinsed with HBSS to remove free bacteria, then fixed with methanol. The

number of phagocytes containing or associated with more than 5 bacteria out of a total of 200 glass-adherent cells was counted microscopically. Adherent cells such as lymphocytes were disregarded. Head kidney cells and MS93003 KG− phenotype cells previously opsonized with immune serum were incubated for 3h at 25℃. Cells were fixed for transmission electron microscopy after 3h by the addition of 2% glutaraldehyde-0.1M cacodylate buffer(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 analyzed using Student's *t*-test. Phagocytosis assay was analyzed using Duncan's multiple range test.

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 KG−, and from 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 295 days after immunization. After challenge with *L. garvieae* MS93003 KG− there were no mortalities among fish immunized with FKC-KG+; 2 died at 65 days among the KG− phenotype vaccinates. No mortalities occurred in any of the vaccinates and control before artificial infection with *L. garvieae*. No bacteria were isolated from survivors in vaccinated fish 14 days after infection. At 358 days 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. garvieae* was recovered from one surviving fish 14 days 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 15 to 135 days after immunization than control but not after 295 days. Agglutinating titres against KG− phenotype cells in fish immunized with FKC-KG− were <1:4 to 1:4 throughout the experiments.

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 i*n vitro* morphology of *Lactococcus garvieae.

L. garvieae ingested in vitro by yellowtail phagocytic cells were seen to be enclosed in a phagosome 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).

Discussion

The antigenic conversion of *Lactococcus garvieae* occurred after several subcultures on KF Streptococcus agar supplemented with 2,3,5-triphenyltetrazolium chloride (TTC). The KG − phenotype (non-agglutinating strain against KG+ antiserum) strains were more

virulent than KG+ cells(Kitao, 1983 and Alim *et al.*, 1996).

A common strategy for bacteria to avoid the host defense system is the production of anti-phagocytic surface components. Capsulated 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 lead to mortalities in fish (Yoshida *et al.*, 1996b). Yoshida *et al.* (1996a) reported similar findings for *L. garvieae* KG− phenotype (capsulated), which was more hydrophilic than the KG+ variant (non-capsulated) 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 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 *L. 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− cells emulsified with adjuvant.

However, the agglutinating titres of KG− phenotype cells were very low in farmed fish compared to titres against 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 *L. 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 month as show 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+.

Appendages were seen extending from the cell surface of *L. garvieae* with some disruption of cell capsule after opsonization with yellowtail immune serum. This finding may indicates 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 capsulated 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 and 1994b) 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.

No mortalities or abnormalities were observed in any of the fish vaccinated in this study. Poppe and Breck (1997) reported severe adhesions between abdominal organs and body wall in Atlantic salmon immunized by intraperitoneal injection of oil-adjuvanted vaccines. This study demonstrated that the injected vaccines of formalin-killed KG− and KG+ cells conferred adequate long-term protection against *L. garvieae* infection without a second booster or need of an adjuvant on vaccinated fish.

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

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

a; MS93003 KG+ phenotype cells were obtained after subculturing MS93003 KG— phenotype cells on Todd-Hewitt agar containing 2,3,5-triphenyltetrazolium chrolide (TTC).

Table 2. *Seriola quinqueradiata* infected with *Lactococcus garvieae*. Fish mortality (numbers of dead fish/numbers of infected fish) during immunization with formalin-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				
	15	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.

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 titres of immune serum against KG− and KG+ phenotype cells were 1:4 and 1:64, respectively

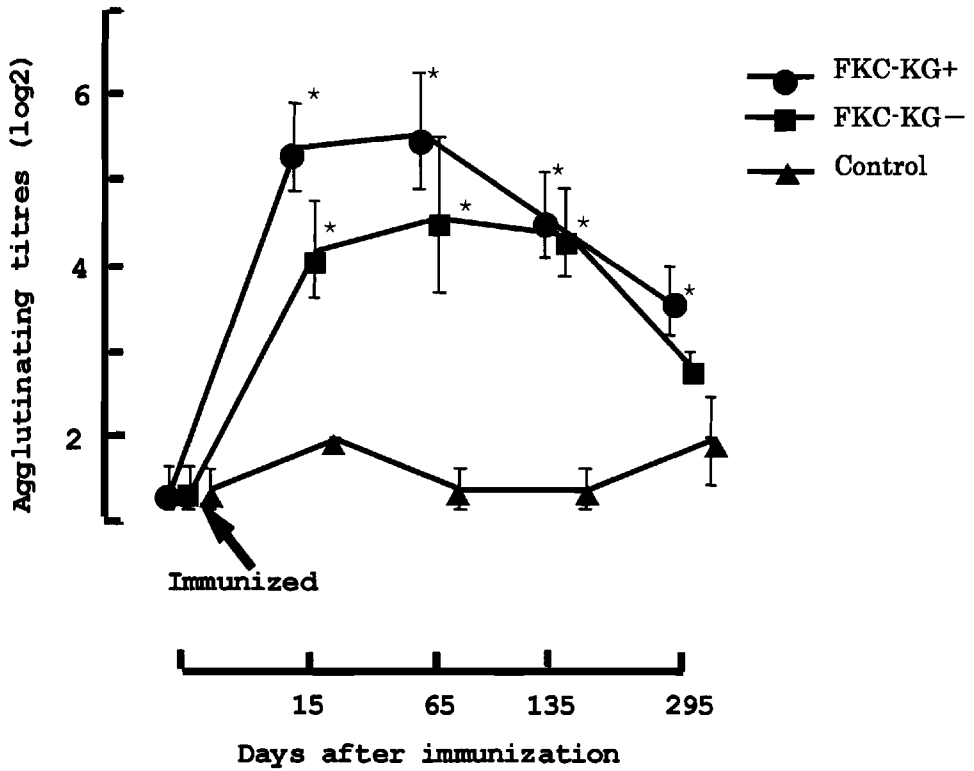

Fig. 1. *Seriola quinqueradiata* immunized with formalin-killed *Lactococcus garvieae* Vaccines. Agglutinating titres against KG+ phenotype cells in serum from fish immunized with MS93003 FKC-KG− phenotype cells, FKC-KG+ phenotype cells or non-treated fish. Agglutinating titres against KG− phenotype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

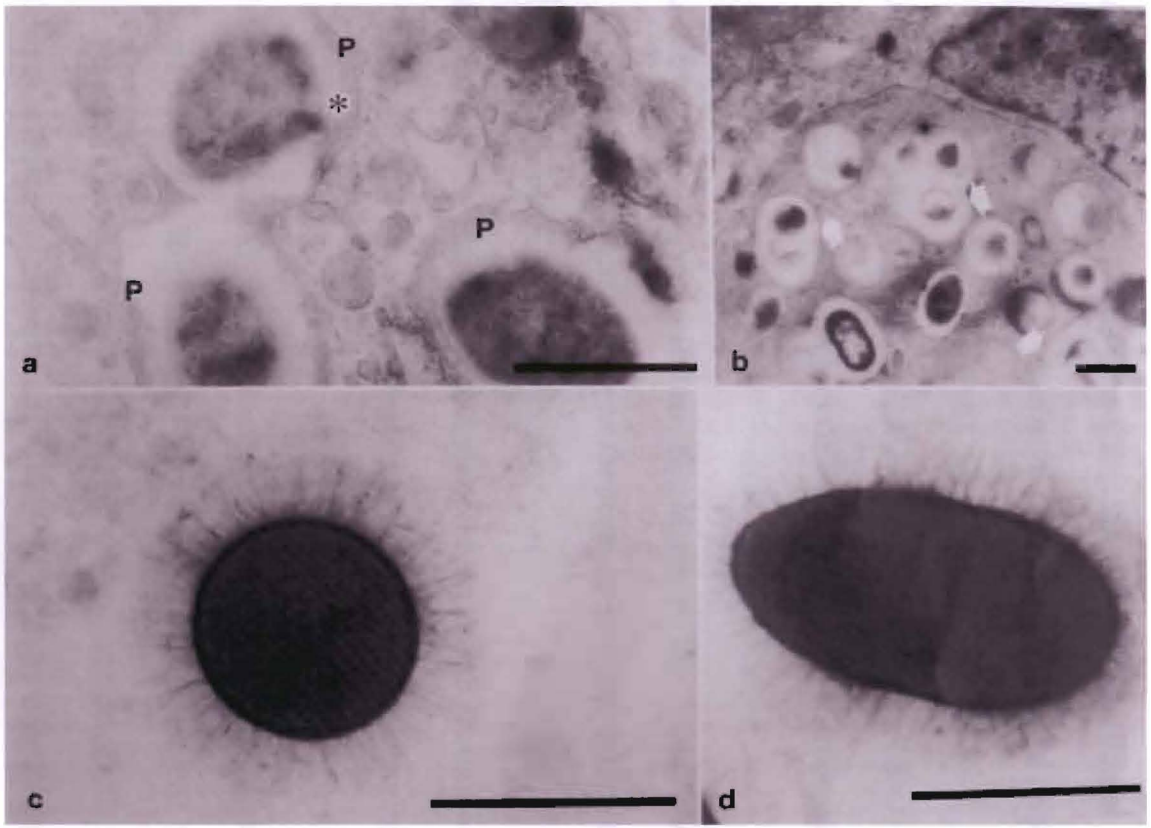

Fig. 2. *Lactococcus garvieae* infecting *Seriola quinqueradiata*. (a) At 3h post phagosytosis assay, *L. garvieae* is found in phagosome (P) with a partial bacteria lysis(*). (b) At 1 d post infection with L. garvieae, fish transplanted with immune serum showed bacterial cells in vacuoles with bacterial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

References

Alim SR, Kawai K, and Kusuda R (1996):Comparative pathogenicity study on antigenically variant strains of *Enterococcus seriolicida.* J. Fish Dis., 19:39-46

Aoki T, Takami K, and Kitao T (1990): Drug resistance in a non-hemolytic Streptococcus sp. isolated from cultured yellowtail, *Seriola quinqueradiata.* Dis. Aquat. Org., 8:171-177

Arduino RC, Murray BE, and Rakita RM (1994a): Roles of antibodies and complement in phagocytic killing of enterococci. Infect. Immun., 62:987-993

Arduino RC, Jacques-Palaz K, Murray BE, and Rakita RM (1994b): Resistance of *Enterococcus faecium* to neutrophil mediated phagocytosis. Infect. Immun., 62:5587-5594

Braun-Nesje R, Kaplan G, and Seljelid R (1982): Rainbow trout macrophages *in vitro*: morphology and phagocytic activity. Develop. Comp. Immunol., 62:281-291

Czuprynski, CJ (1988): Bacterial evasion of cellular defense mechanisms: an overview. In: Roth J.A (ed) Virulence mechanisms of bacterial pathogens. American Society for Microbiology, Washington, D.C., p141-160

Eldar A, Ghittino C, Asanta L, Bozzetta E, Goria M, Prearo M, and Bercovier H (1996): *Enterococcus seriolicida* is a junior synonym of *Lactococcus garvieae*, a causative agent of septicemia and meningoencephalitis in fish. Curr. Microbiol., 32:85-88

Harvey BS, Baker CJ, and Edwards MS (1992): Contributions of complement and immunoglobulin to neutrophil-mediated killing of enterococci. Infect. Immun., 60:3635-3640

Iida T, Wakabayashi H, and Egusa S (1982): Vaccination for control of streptococcal disease in cultured yellowtail. Fish pathol., 21, 33-38.

Kitao T (1982): The methods for detection of *Streptococcus* sp. causative bacteria of streptococcal disease of culture yellowtail, *Seriola quinqueradiata*, especially, their cultural, biochemical and serological properties. Fish Pathol., 17:17-26

Kitao T (1983): Strain variation associated with pathogenesis of *Streptococcus* sp., the causative agent of streptococcosis in cultured yellowtail, *Seriola quinqueradiata*. Proc 2nd Natl Pacific Aquaculture Symposium, Tokyo and Shimizu, Japan, 1983, Tokai University, p196-210

Kitao T (1993): Streptococcal infection. In:Inglis V, Roberts RJ, Bromage NR (eds) Bacterial diseases of fish. Blackwell Scientific Publications, Oxford, p196-210

Kusuda R, Kawai K, Salati F, Banner CR, and Fryer JL (1991): *Enterococcus seriolicida* sp. nov., a fish pathogen. Int. J. Syst. Bacteriol., 41:406-408.

Poppe TT, and Breck O (1997): Pathology of atlantic salmon Salmo salar intraperitoneally immunized with oil-adjuvanted vaccine. A case report. Dis. Aquat. Org., 29:219-226

Roberson BS (1990): Bacterial agglutination. In:Stolen JS, Fletcher TC, Anderson DP, Roberson BS, van Muiswinkel WB (eds) Techniques in fish immunology. SOS Publications, Fair Haven, NJ, p81-86

Sato H, Ninomiya M, Kawai K, and Kusuda R (1996): Protection and opsonic activity in the serum of yellowtail *Seriola quinqueradiata* immunized with *Enterococcus seriolicida* formalin-killed cells. Nippon Suisan Gakkaishi.,62(6):939-940 (in Japanese with English abstract)

Teixeira LM, Merquior VLC, Vianni MCE, Carvalho MGS, Fracalanzza SEL, Steigerwalt AG, Brenner DJ, and Facklam RR (1996): Phenotypic and genotypic characterization of atypical *Lactococcus garvieae* strains isolated from water buffalos with subclinical mastitis and confirmation of *L. garvieae* as a senior subjective synonym of *Enterococcus seriolicida*. Int. J. Syst. Bacteriol., 46:664-668

Williams P (1988): Role of the cell envelope in bacterial adaptation to growth *in vivo* in infections. Biochimie.,70:987-1011

Yoshida T, Eshima T, Wada Y, Yamada Y, Kakizaki E, Sakai M, Kitao T, and Inglis V (1996a): Phenotypic variation associated with an anti-phagocytic factor in the bacterial fish pathogen *Enterococcus seriolicida*. Dis. Aquat. Org., 25:81-86

Yoshida T, Yamada Y, Sakai M, Inglis V, Xie XJ, Chen SC, and Kruger R (1996b): The association of the cell capsule with anti-opsonophagocytosis in β -hemolytic *Streptococcus* spp. isolated from rainbow trout, *Oncorhynchuss mykiss*. J. Aquat. Anim. Health, 8:181-186

Yoshida T, Endo M, Sakai M, and Inglis V (1997): A cell capsule with possible involvment in resistance to opsonophagocytosis in *Enterococcus seriolicida* from yellowtail, *Seriola quinqueradiata*. Dis. Aquat. Org., 29:233-235

Chapter III

Cell-surface properties of *Lactococcus garvieae* strains and their immunogenicity in the yellowtail *Seriola quinqueradiata*

Introduction

Lactococcus garvieae is a serious bacterial pathogen of yellowtail, *Seriola quinqueradiata* and amberjack, *Seriola dumerillei* in Japan (Kusuda *et al.*,1991; Kitao,1993). It has also been isolated from rainbow trout, *Oncorhynchus mykiss* in Italy (Eldar *et al.*, 1996 and 1999) and Australia (Carson *et al.*,1993; Schmidtke and Carson,1999), and from the prawn, *Macrobranchium rosenbergii* in Taiwan(Chen *et al.*, 2001). *L. garvieae* isolated from *S. quinqueradiata* has been divided into non-agglutinating (KG − phenotype) and agglutinating (KG+ phenotype) phenotypes using anti-KG+ phenotype serum (Kitao, 1982; Yoshida *et al.*, 1996 and 1997; Ooyama *et al.* 1999). The KG− phenotype was agglutinated by anti-KG− phenotype cell serum (but not by antisera to the KG+ phenotype ,KG7409). However, KG+ phenotype strains were agglutinated with antisera to both KG+ and KG− phenotypes (Kitao, 1982 and 1993). Furthermore, in KG− phenotype cells incubated with anti-KG− phenotype serum and stained with ruthenium red, cell capsules adjacent to the cell wall were visible by electron microscopy. This capsulated phenotype strain is more virulent to the yellowtail than non-capsulated strain (Alim *et al.*, 1996). Therefore these capsules are thought to play roles in the pathogenicity of *L. garvieae*

infection, possibly by increasing resistance to fish phagocytosis (Yoshida *et al.*, 1996 and 1997).

Control of *L.garvieae* infection in yellowtail culture has depended on chemotherapy with macrolides. The identification of multiple drug-resistant strains has indicated future problems in control (Aoki *et al.*, 1990). Recently, oral and injectable vaccines against *L. garvieae* infection in *S. quinqueradiata* have been developed and commercialized in Japan. Experimental vaccination against *L. garvieae* has been described (Iida *et al.*, 1982; Sato *et al.*, 1993) and has been reported to provide immunity and increased opsonic activity in the fish. However, no detailed information of the antigenicity of *L. garvieae* phenotypes nor the duration of the immunity was given. The protective mechanisms of the *L. garvieae* vaccine remains unknown.

In a previous study, Ooyama *et al.* (1999) reported that formalin-killed *L. garvieae* KG−phenotype (capsulated phenotypes) cells and KG+ phenotype (non-capsulated) cells induced strong immunity in *S. quinqueradiata* against artificial infection and long-lasting agglutinating titres against non-capsulated cells (avirulent KG+ phenotype). Furthermore appendages (fimbrie-like structures) were seen extending from the cell surface of *L. garvieae* KG− phenotype, with some destruction of the cell capsule after opsonization with yellowtail immune serum. Both formalin-killed cells of the KG− and KG+ phenotypes were effective against infection with KG− virulent phenotype cells, suggesting that the antigen providing immunity against *L. garvieae* infection is located on the cell surface or projects into the

cell capsules from the surface, and is not present in the cell capsules themselves.

Recently, some strains of *L. garvieae* KG+ phenotype were found that induced a weak immune response in fish against capsulated virulent KG− phenotype infection. The aim of the present study was to re-examine the cell-surface properties of various *L. garvieae* strains and compare the immune response in *S. quinqueradiata* to these strains.

Materials and Methods

Bacterial strains.

Bacterial strains are listed in Table 4. All were cultured in Todd-Hewitt broth (THB; Difco, Detroit, Michigan, USA) or on agar (THA). Immune sera against the NG8206 KG+ phenotype (non-capsulated cells) and KG9408 KG − phenotype (capsulated cells) were raised in rabbits, as described by Yoshida *et al.* (1996). The KG+ phenotypes (NG8206, NSS9310 and MS93003) stemmed from a subculture of the KG − parent on THA supplemented with 2,3,5-triphenyltetrazolium chloride (TTC) as previously described by Ooyama *et al.* (1999).

The yellowtail fish *Seriola quinqueradiata* used in the experiment were bred at Miyazaki Experimental Fisheries Station, Aoshima, Miyazaki, Japan, and kept in concrete tanks with sand-filtered sea water. The fish were fed commercial dried pellets once a day. Before the experiment, the fish (n=10) were subjected to bacterial examination to determine the presence of *Lactococcus garvieae*.

The bacteria were cultured in THB at 25℃ without shaking for 24h. Cells were harvested by centrifugation and suspended in sterile saline (0.85% NaCl). Serial dilutions were prepared in saline, and viable counts were determined by plating on THA. Virulence was tested with 10 fish(approximately 20 g) per dilution inoculated intraperitoneally with 0.1 ml of each bacterial suspension: 3.2×10^3, 3.2×10^4 and 3.2×10^5 CFU ml^{-1} for KG9408 (KG− phenotype cells), 5.2×10^5, 5.2×10^5 and 6.2×10^5 CFU ml^{-1} for NSS9310, MS93003 and NG8206 (KG+ phenotype cells), respectively. The fish were maintained at 24 to 25℃ for 14 days in separate tanks. Non-treated fish were injected with saline as controls. Virulence was expressed as the lethal dose 50 (LD_{50}) (Reed and Muench, 1938).

Transmission electronmicroscopy (TEM).

Transmission electronmicroscopy (TEM) was performed as described by Yoshida *et al.* (1997). Briefly, *L. garvieae* KG− and KG+ (KG9408, MS93003 and NSS9310) were grown overnight in 10ml of THB, diluted 1:100 in fresh THB, and incubated for an additional 5 h at 25℃. Bacteria were washed with phosphate-buffered saline (PBS) and fixed with 2.5% glutaraldehyde. The cells were washed 3 times with PBS and resuspended in 10ml of rabbit anti-KG− serum (agglutinating titre against KG9408, KG− phenotype cells; 1:1280) diluted 200 times with PBS and incubated for an additional hour before staining with 0.15% ruthenium red in 0.1M cacodylate buffer, pH 7.4 for 2h. Bacteria were washed 3 times with PBS, embedded in 3 % agarose, fixed with 2% osmium tetroxide, washed 5 times with cacodylate buffer, and

dehydrated with ethanol. Cells were embedded in Quetol 652 (Nishin EM, Tokyo Japan). Thin sections were cut (60nm), post-stained with uranyl acetate and lead acetate, and observed by transmission electron microscopy (Hitachi-H4800Mu, Japan) at an accelerating voltage of 100 KV. For investigation of fimbriae-like structures on the cell surface, bacterial strains were cultured in 10 ml of normal filter-sterilised yellowtail serum (100%) at 25℃ for 48h, were washed twice with PBS, and fixed with 2 % glutarldehyde. Samples for TEM were treated as described above.

Bacteriophage susceptibilities.

L. garvieae bacteriophage strains PLgY-16, PLgY-30 and PLgW-1 were used in this study (Park *et al.*, 1997 and 1998). The phages were propagated on *L. garvieae* NSS9310 (indicator bacterium) by the double agar-overlay method (Paterson *et al.*, 1969), and the susceptibilities of *L. garvieae* strains were then assessed by plaque formation using the same method.

Vaccination and challenge test.

KG9408, MS93003 and NSS9310 strains were cultured in THB and killed by addition of a final concentration of 0.3% formaldehyde. Bacterial cells were washed 3 times with phosphate-buffered saline and adjusted to 1.0 at an OD of 660 nm. Yellowtail (200 to 250g, n=26×3) were immunized with each bacterin (KG9408, MS93003 and NSS9310); 14 days later, each immunized fish (n=16×3) were injected intraperitoneally with KG9408 KG

− phenotype cells at a density of 2.5×10^5 cells $fish^{-1}$. The fish was monitored daily for 14 days, and individuals that died were subjected to bacteriological examination to verify the cause of death. Immunized fish (n=10×3) without infection were bled to provide immune serum 14 days after immunization. Non-treated control fish(n=26) were injected with saline;14 days later, a further 16 fish were infected with the same dose of KG9408 (KG− phenotype cells). Normal serum isolated from fish (n=10) with no infection 14 days after sham-injection (saline). Fish sera from each immunized and control fish were filtered through a 0.45 μm pore-size filter (Sartorius), and kept at −80℃ until use. Immune sera from each were pooled for all immunized fish and also for all non-treated fish, respectively, for passive immunization tests and immunoblott analysis. Agglutinating titres of immune sera against KG9408, MS93003 and NSS9310 were measured by a microplate assay according to Roberson (1990). Throughout the experiment, from immunization to the challenge test, the water temperature was between 24 and 26℃.

Passive immunization.

Yellowtail(75 to 105g; n=13×3) were inoculated intraperitoneally with 3 ml of immune serum from fish immunized with KG9408, MS93003, or NS9310 formalin-killed cells. Control fish (n=13) were given 3 ml of normal yellowtail serum from non-treated healthy fish (200 to 250g; n=10). The fish were challenged intraperitoneally 30 h later with KG9408 KG− phenotype cells at a density of 2.5×10^4 cells $fish^{-1}$, and were monitored for 14 days. At

the end of this period, all fish were subjected to bacteriological examination for the presence of *L. garvieae* in the brain and kidney; 30 h after infection, 3 fish were also sampled for bacteriological counts in the blood, spleen and brain using plate counts. These organs and blood were aseptically sampled, and homogenized in phosphate-buffered saline supplemented with heparin (100 units ml^{-1}) diluted, and spread over THA plates supplemented with 0.025% TTC. Colony-forming units (CFU) of *L. garvieae* were counted after 72 h incubation at 25℃ and expressed as CFU g^{-1} for brain and spleen, or CFU ml^{-1} for the blood. Throughout the experiment, from passive immunization to the infection test, the water temperature was between 24 and 26℃.

Heat stability of bacterin.

KG9408 formalin-killed cells were heated to 100 or 121℃ for 15 min, or left non-treated (kept at 4℃) to compare the heat stability of the antigens that conferred the protection on the fish. Fish(45 to 60g;n=10×3) were immunized with each heat-treated or non-treated bacterial cells. Control fish (n=10) were injected intraperitoneally with saline. Fish were challenged intraperitoneally with KG9408 cells at a density of 2.1×10^{6} CFU $fish^{-1}$ 14 days after immunization, and were monitored for a further 14 days. Throughout the experiment, from immunization to the infection test, the water temperature was between 24 and 26℃.

Whole-cell protein and immunoblotting analysis.

Whole-cell protein extracts were prepared as described by Carson *et al.* (1993). Proteins were separated by SDS-polyacrylamide gel electrophoresis using 12.5% acrylamide-separating gel with 2.5% stacking gel (Laemmli 1970). Proteins were blotted onto tranfer membranes (Immobilon™-P Transfer Membrane, Millipore) as described by Towbin *et al.*, (1979). Immunoblotting analysis of protein extracts from KG9408, MS93003, and NSS9310 were performed using rabbit anti-KG9408 and anti-MS93003 sera, and yellowtail anti-KG9408 and anti-MS93003 sera. A rabbit anti-yellowtail immunoglobulin serum was prepared according to Smith (1992). Yellowtail anti-KG9408 and anti-MS93003 sera, rabbit anti-yellowtail immunoglobulin, and rabbit anti-KG9408 and anti-MS93003 sera were diluted 1:100, 1:1000 and 1:500,respectively. The antibodies bound to proteins immobilized on the membranes were detected with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG; Sigma) and developed by addition of 5-bromo-4-chloro-3 indolyl phosphate as the substrate, and nitroblue tetrazolium salt as the developer.

Statistical analysis.

Statistical analyses of the protective efficacy of the vaccines and passive immunizations were performed by Fisher's protected least-squares differences test. The significance of the different plaque sizes of bacteriophage PLgW-1 on KG9408, MS93003 and NS9310 was analyzed using Welch's test.

Results

The lethal dose 50, cell capsulation and fimbriae-like structures.

The LD_{50} of each bacterial strain is shown in Table 5. Strains with a highly developed capsule KG9408(KG− phenotype cells) were more virulent to the fish than NG8206, MS93003 and NSS9310 (KG+ phenotype cells). Cell capsules ranging from 100 to 200nm for KG9408 and micro-capsule (approximately 10nm) for MS 93003 were observed after treatment with rabbit anti-KG− phenotype serum. However, no capsule was observed for NSS9310 even after treatment with immune serum. Fimbriae-like structures on the surface of KG9408 and MS93003 were observed when the cells were cultured in yellowtail serum. NSS9310 showed no surface visible components when the cells were cultured in fish serum (Fig. 3).

Bacteriophage susceptibilities.

MS93003 and NSS9310 were highly susceptible to bacteriophages PLgY-16 and PLgW-1 ,but KG9408 was not susceptible. Although the titres of bacteriophage PLgY-30 against KG9408, MS93003, and NSS9310 were similar (Table 6), the plaque sizes of PLgW-1 on KG9408 (0.5mm±0.2) were significantly different ($p<0.05$) from MS93003 (1.4mm±0.5) and NSS9310 (1.4mm±0.7).

Efficacy of vaccines.

The protective efficacy of vaccines against artificial infection of KG9408-virulent KG− phenotype cells is shown in Table 7. Significantly

higher protection was recorded in fish immunized with formalin-killed KG9408 and MS93003 cells compared to non-treated fish ($p<0.01$) and fish immunized with NSS9310 formalin-killed cells ($p<0.05$). However, some protection was still provided by the NSS9310 vaccin compared to non-treated control fish ($p<0.05$).

Efficacy of passive immunization against artificial challenge with *L. garvieae.*

The agglutinating titres of pooled yellowtail immune sera against KG9408, MS93003 and NSS9310 are shown in Table 8. The agglutinating titres of immune sera against KG9408 ranged from <1:4 to 1:8, while the titres of immune sera against MS93003 and NSS9310 were 1:64 to 1:256. Aggutinating titres of serum from non-treated fish against KG9408, MS93003 and NSS9310 were not detected. No mortailities were observed in fish passively immunized with anti-KG9408 or anti-MS93003 sera, but fish given anti-NSS9310 were not completely protected against KG9408 infection. Bacteriological examination of surviving fish which had been passively immunized against KG9408 or MS93003 indicated complete eradication of the bacteria. In contrast, bacteria were detectable in the kidney and brain of fish passively immunized against NSS9310 (Table 9). Bacteria were eradicated rapidly from the blood, spleen and brain of fish passively immunized against KG9408 or MS93003, yet were detectable in the blood and spleen but not the brain of fish treated with anti-NSS9310 serum (Table 10).

Stability of KG9408 bacterin to heat.

Treatment of KG9408 formalin-killed cells at 121℃ for 15 min decreased the protection of the fish against homologous bacterial infection (mortality=80%). Parallel groups of fish immunized with the KG9408 treated at 100℃ for 15 min, or with non-heat treatment (kept at 4℃) and a control group without immunization had mortality rates of 20, 0 and 90%, respectively.

Immunoblot analysis of whole-cell protein extracts with rabbit and fish anti-KG9408 and anti-MS93003 immune sera.

Immunoblot analysis of proteins extracted from KG9408, MS93003, NSS9310 and *Lactococcus lactis* ATCC19435 strains using rabbit anti-KG9408 and anti- MS93003 serum, and yellowtail anti-KG9408 and anti-MS93003 serum are shown in Fig. 4. Two protein bands, ranging from 30 to 35 kDa and 15 to 20 kDa were missing in the NSS9310 strain compared with the protein profiles of the KG9408 and MS93003 strains using rabbit antiserum against KG9408 and MS93003 (Fig. 4-A). Immunoblotting analysis showed that although yellowtail anti-KG9408 immune serum detected protein bands of approximately 30 to 35 kDa and 45 to 50 kDa in the extracts from strains KG9408 and MS93003, the bands were not observed in NSS9310 strain treated with yellowtail anti-KG9408 serum. With yellowtail anti-MS93003 serum, an increase in staining intensity was observed for detectable antigens of approximately 30 to 35 kDa and 40 to 50 kDa in KG9408 and MS93003 compared with NSS9310 (Fig.4 B). Both rabbit and yellowtail anti- KG9408 and anti- MS93003 sera detected several

antigens of *L. lactis* ATCC19435 (Fig. 4 A,B).

Discussion

Antigenic conversion from the KG− to KG+ phenotype in *Lactococcus garvieae* occurred after several subcultures on agar media supplemented with TTC. The KG − phenotype (non-agglutinating strain against KG+ antiserum) strains were capsulated and more virulent to fish than the KG+ phenotype (Alim *et al.*, 1996). No cell capsules could be seen by the Indian ink or the 'quellung' method (Yoshida *et al.*, 1997), but after treatment of KG − phenotype cells with antiserum raised against the capsulated strain, well-developed capsules were seen on the surface of *L. garvieae*. Furthermore, KG+ antigens were detected only around the cell surface but not in cell capsules of *L. garvieae*, whereas KG− antigens were detected spreading over the capsule. Cell capsules inhibited cell agglutination with serum raised against KG+ phenotype cell (non-capsulated cells) and was possibly involved in resistance to fish phagocytosis (Okada *et al.*, 2000). In a previous investigation, formalin-killed KG − and KG+ phenotype cells induced high protection in *S. quinqueradiata* against artificial infection with capsulated virulent strain (KG− phenotype cells) with immunity lasting at least 10 month. Therefore, it was speculated that antigens associated with the immunity against *L. garvieae* were located on the bacterial surface not in the capsules (Ooyama *et al.*, 1999).

In this study, formalin-killed cells of KG9408 and MS93003 also induced a high level of protection in fish against capsulated virulent cells, although the

immunity in fish immunized with NSS9310 KG+ phenotype cells was only partial. Passive immunization of yellowtail with yellowtail antisera raised against KG9408 or MS93003 strains also showed rapid bacterial clearance from blood and spleen, and conferred high immunity on the fish. Viable bacteria were recovered from the blood and spleen of fish treated with yellowtail anti-NSS9310 strain serum, but at lower levels than from fish injected with non-immune fish serum. These results support previous findings that humoral immunity plays an important role in protection against infection by *L. garvieae* (Ooyama *et al.*,1999; Barnes *et al.*,2002a,b). They suggest ,moreover, that complete immune protection against the infection could be induced with NSS9310 antigens in combination with other antigens.

In a previous study, the MS93003 strain was classified as KG+ phenotype cells with no cell surface components (Ooyama *et al.*, 1999). However, in detailed observation, a micro-capsule was seen on MS93003 after treatment with rabbit anti-KG − phenotype serum. Furthermore, fimbriae-like cell surface components were observed after culture in fish normal serum. The LD_{50} and susceptibility of MS93003 to bacteriophages were similar to those of NSS9310 KG+ phenotype cells, suggesting that a micro-capsule is not as strongly related to degree of virulence as is a well-developed cell capsule. The antigenicity of KG9408 and NSS9310 were differentiated by a cell agglutination test using yellowtail anti-NSS9310 serum, but a micro-capsulated strain, MS93003 could not be differentiated from non-cell capsulated strain, NSS9310 using antisera to NSS9310 and

MS93003. A thin layer inside the well-developed cell capsule on KG9408 strain could be seen by TEM. These results suggest the possibility of two immunologicaly different types of cell capsule in *L. garvieae*, the micro-capsule and well-developed capsule in *L. garvieae*. In *in vivo* passage of the MS93003 strain in *S. quinqueradiata*, the cells remained micro-capsulated, did not change to the well-developed form and showed same levels of LD_{50} (data not shown).

Attachment to host cells is important in initiating infection by pathogens (Marques *et al.*, 1984). Bacterial lectins and fimbriae are thought to participate in bacterial attachment to animal cells or erythrocytes (Nakasone and Iwanaga, 1993). *Enterococcus faecalis* strains carry thin peritrichous fimbriae on a proportion of their cells, and the percentage of fimbriated cells varies throughout the growth cycle (Handley and Jacob, 1981). The major virulence factor of Group A (Streptococci) is M protein, a fibrillar surface molecule that protects the bacteria from being ingested and killed by the host's phagocytic cells (Lancefield,1962 ;Phillips *et al.*,1981). In a previous study (Ooyama *et al.*, 1999), fimbriae-like cell surface components were seen on the KG9502 capsular virulent strain of *L. garvieae* incubated with yellowtail immune serum. It was difficult to differentiate fimbriae from cell capsules after treatment of cells with rabbit immune serum against capsulated cells. In this study, fimbriae-like cell surface components of MS93003 and KG9408 strains were clearly demonstrated in yellowtail serum cultures. Some factor(s) in the fish serum may enhance the presence of cell-surface components of *L. garvieae*.

However, the LD_{50} of Strain MS93003, carrying fimbriae-like surface components, was similar to that of NSS9310 with no cell-surface components. The role of fimbriae-like cell surface components on *L. garvieae* is not yet known, and further investigation is needed.

Virulent bacteriophages have been isolated and used in the typing of *L. garvieae* strains isolated from yellowtail (Park *et al.*, 1997 and 1998). In the present study, the KG+ phenotypes of NSS9310 and MS93003 differed in phage susceptibility to PLg-16 from the KG9408(KG− capsular stain). Furthermore, the plaque sizes of PLgW-1 on NSS9310 and MS93003 differed from those of KG9408. KG9408 susceptibility to PLgY-16 could be induced after several subcultures on agar media supplemented with TTC (data not shown). It is suggested that these changes in phage susceptibility may be influenced by the size of the capsule.

Humoral immunity plays an important role in the protection of yellowtail from *L. garvieae* infection (Ooyama *et al.*, 1999). Antigens eliciting protection in fish were reduced when the bacterial cells were kept at 121℃ for 15 min, indicating that they are relatively heat-labile. However, the well-developed cell capsule associated with virulence might not be needed to induce protection in fish against *L. garvieae* infection. This possibility was investigated by carrying out a protein analysis of virulent KG9408 and non-virulent MS93003 and NSS9310 strains. Immunoblotting analysis using rabbit anti-KG9408 and anti-MS93003 serum showed the loss of 2 protein antigens (15 to 20 kDa and 30 to 35 kDa) in NSS9310 compared to KG9408 and MS93003. Hirono *et al.* (1999) reported 5 different clones from a gene

library of *L. garvieae* KG− phenotype cells. The reaction of recombinant proteins expressed by recombinant *Escherichin coli* JM109 with anti-KG− and anti-KG+ specific rabbit serum indicated that the molecular size of immunologically-detected KG− specific proteins of these clones was 25, 26, 28, 30 and 13 kDa. It is possible that there is a deficiency of immunologically-detectable proteins in NSS9310 compared with MS93003 and KG9408 when treated with rabbit immuno serum, and this may provide incomplete protection to fish. An analysis of protein profiles from KG9408, MS93003 and NSS9319 by yellowtail anti-KG9408 and anti-MS93003 serum also revealed that several protein bands were missing from NSS9310 stain compared to KG9408 and MS93003. Although analysis with rabbit antiserum and yellowtail antiserum revealed that some proteins were missing from NSS9310, the molecular sizes of the missing bands did not coincide with each other. The immuno recognition of rabbit serum against *L. garvieae* could be different from the reaction of yellowtail serum. Formalin-killed KG+ phenotype cells could induce high agglutinating tetres against the homologous cells in yellowtail serum, but not against KG− phenotype cells. However, rabbit immunized with KG− phenotype cells induced high agglutinating titres in serum against the homologous strain(Kitao,1982; Yoshida *et al.*,1996).

Alim *et al.* (2001) reported that cell-surface immunoprotective, 96 kDa glycoprotein from a non-agglutinating strain of *Enterococcus seriolicida* (KG − phenotype) is specific to an antigen on the cell surface of KG− phenotype cells. However, the present study could not identify the 96kDa protein band

using the yellowtail anti-KG− serum. Immunoprotective antigens are still unidentified. Further investigation determining the molecular size of antigens are needed. Rabbit and yellowtail anti-KG9408 and anti-MS93003 sera also detected antigens in *Lactococcus lactis* ATCC19435. Formalin-killed *L. lactis* ATCC19435 induced some degree of protection in yellowtail against KG9408, but this was incomplete (data not shown). Therefore, antigens common to *L. garvieae* and *L. lactis* ATCC19435 may induce some degree of immunity in the yellowtail *Seriola quinqueradiata.* These results suggest that several antigens are needed to confer complete protection against *L. garvieae* infection.

Table 4. *Lactococcus garvieae* strains used in this study

Strain	Year	Sources (Prefectures)	Phenotypes Anti-NG8206 KG+ serum	References
NG8206	1982	Nagasaki	KG+	Yoshida *et al.* (1996 and 1997), Ooyama *et al.*(1999), Okada *et al.*(2000)
MS93003	1993	Miyazaki	KG+	Ooyama *et al.* (1999)
NSS9310	1993	Nagasaki	KG+	This study
KG9408	1994	Kagoshima	KG−	This study

Table 5. *Lactococcus garvieae*. Lethal dose 50 (LD_{50}) in the yellowtail *Seriola quinquerdiata* , cell capsulation, and fimbriae-like structures of different bacterial strains.

Strain	LD_{50}	Capsule	Fimbriae-like structure
NG8206	$>1.0\times10^{5}$	N.D	N.D
MS93003	$>1.0\times10^{5}$	Micro-capsule	+
NSS9310	$>1.0\times10^{5}$	N.D	N.D
KG9408	5.2×10^{3}	100-200nm	+

+:present; N.D:not detected

Table 6. *Lactococcus garvieae*. Bacteriophage susceptibilities of different strains

Strain	Bacteriophage titre		
	PLgY-16	PLgY-30	PLgW-1
NSS9310	2.0×10^9	7.0×10^9	2.8×10^9
MS93003	9.0×10^9	2.2×10^9	9.6×10^9
KG9408	$<1.0 \times 10^5$	3.1×10^9	$<1.0 \times 10^5$

Table 7. *Seriola quinqueradiata* infected with *Lactococcus garvieae* KG9408. Fish mortality (numbers of dead fish/numbers of infected fish) in fish immunized with formalin-killed NSS9310, MS93003 and KG9408 strains of *L. garvieae*. Results were significantly different from those for non-treated fish (a;$p<0.01$, b;$p<0.05$) and fish immunized with NSS9310 (c;$p<0.05$).

Vaccine	Fish mortality
NSS9310	8/16 [b]
MS93003	0/16 [a,c]
KG9408	0/16 [a,c]
Non-treated	14/16

Table 8. Agglutinating titres of *Seriola quinqueradiata* immune sera raised against *Lactococcus garvieae* formalin-killed strains.

Antigen	Serum from fish immunized with		
	NSS9310	MS93003	KG9408
NSS9310	1:128	1:256	1:64
MS93003	1:256	1:256	1:128
KG9408	<1:4	1:4	1:8

Table 9. *Seriola quinqueradiata.* Efficacy of passive immunization of yellowtail against *Lactococcus garvieae* infection. Fish mortality: numbers of dead fish/numbers of infected fish, in fish treated with antiserum to KG9408, MS93003 and NSS9310. Survivors *L. garvieae*-positive: numbers of survivors in which *L. garvieae* was detected in kidney and brain after sacrifice; results were significantly different from fish passively given normal serum(*$p<0.05$).

Implanted serum	Fish mortality	Survivors *L. garvieae*-positive
KG9408	0/10*	0/10
MS93003	0/10*	0/10
NSS9310	3/10	2/7
Normal serum	6/10	1/4

Table 10. *Seriola quinqueradiata.* Bacterial counts in blood, spleen and brain of fish passively immunized with yellowtail anti-NSS9310, anti-MS93003 and anti-KG9408 sera.

Antiserum	Fish-no.	Numbers of viable bacteria		
		Blood (CFU ml^{-1})	Spleen (CFU g^{-1})	Brain (CFU g^{-1})
NSS9310	1	2.0×10^{2}	N.D	N.D
	2	2.0×10^{2}	3.0×10^{3}	N.D
	3	3.0×10^{3}	2.0×10^{3}	N.D
MS93003	1	N.D	N.D	N.D
	2	N.D	N.D	N.D
	3	N.D	N.D	N.D
KG9408	1	N.D	N.D	N.D
	2	N.D	N.D	N.D
	3	N.D	N.D	N.D
Normal serum	1	7.5×10^{5}	1.3×10^{4}	1.3×10^{5}
	2	1.4×10^{5}	1.0×10^{5}	3.0×10^{4}
	3	5.4×10^{5}	N.D	N.D

N.D; not detectable

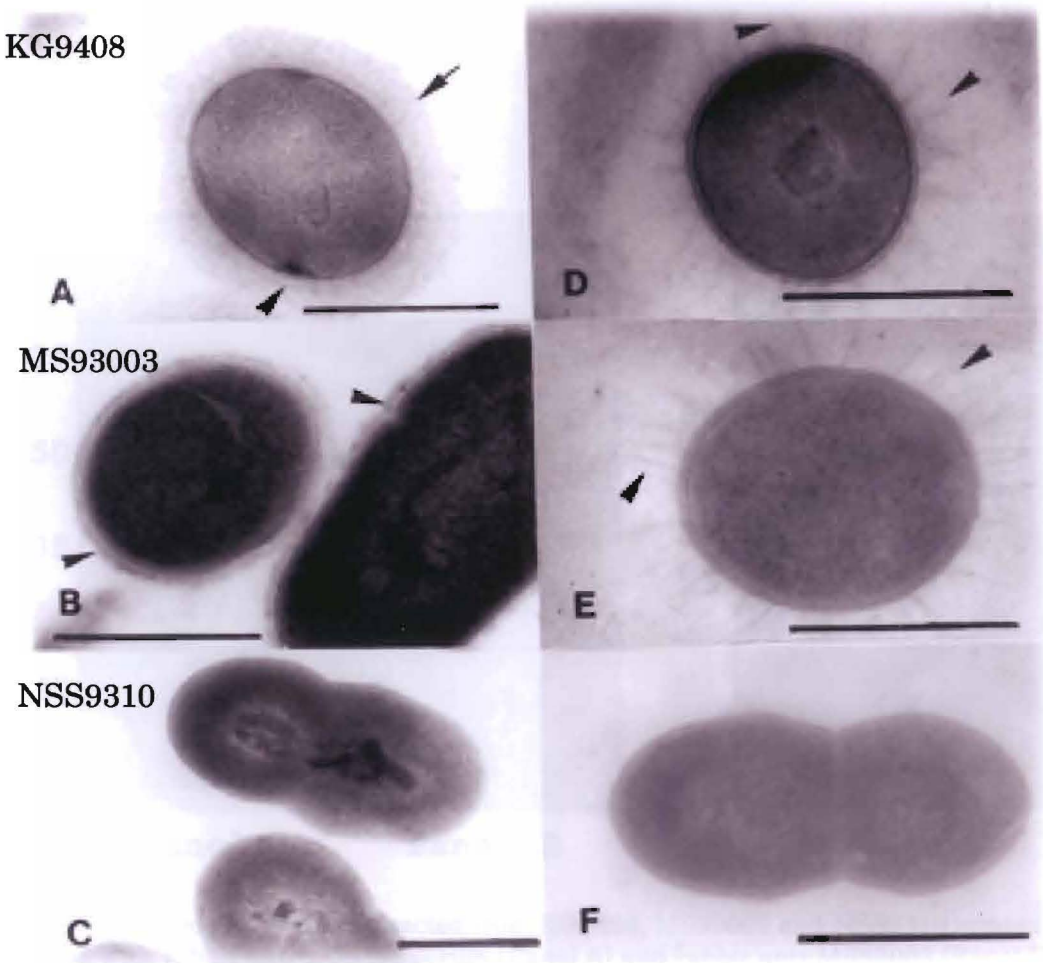

Fig. 3. *Lactococcus garvieae*. Transmission electron microscopy (TEM) of KG9408, MS93003 and NSS9310 strains. (A-C) Cells treated with antiserum raised against capsulated KG9408 KG− phenotype cells; (D-F) cells cultured in *Seriola quinqueradiata* normal serum. (A) Cell, showing well-developed cell capsule (arrow) with faint inside layer (arrowhead); (B) micro-capsule surrounding the cell wall of MS93003 (arrowheads); (C) NSS9310, no cell capsule or micro-capsule visible; (D, E) fimbriae-like structures (arrowheads) projecting from cell surface of KG9408 (D) and MS93003 (E); (F) NSS9310, no cell surface components visible even after culturing in fish normal serum. Scale bars=1 μ m

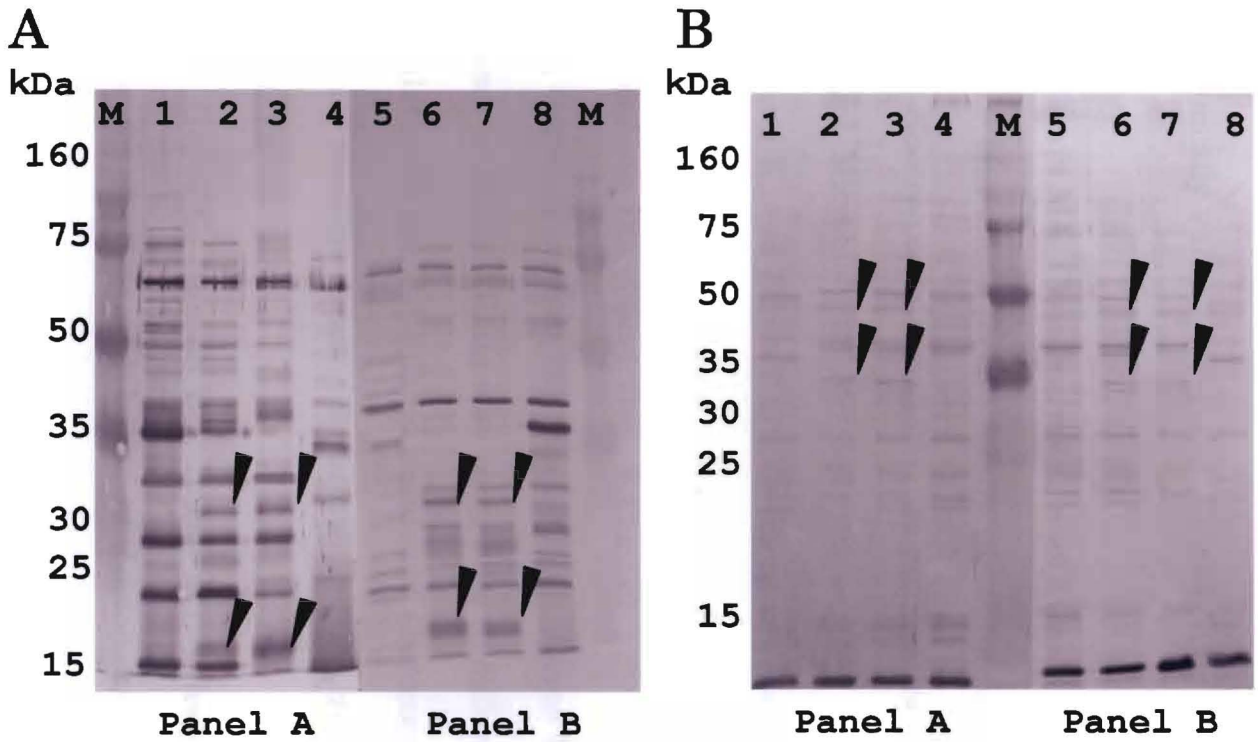

Fig.4. *Lactococcus garvieae*. Detection of proteins extracted from KG9408, MS93003 and NSS9310 strains by immunoblotting. (A)Proteins on filters reacted with rabbit anti-KG9408 serum (Panel A) and rabbit anti-MS93003 (Panel B); Lane M: Marker proteins; Lanes 1 and 8:NSS9310; Lanes 2 and 7: MS93003; Lane 3 and 6: KG9408; Lanes 4 and 5: *L. lactis* ATCC19435; 2 protein bands of approximately 15 to 20and 30 to 35kDa can be seen in KG9408 and MS93003, but not in NSS9310 (arrowheads). (B) Proteins on filters reacted with yellowtail anti-KG9408 serum (Panel A) and yellowtail anti-MS93003 serum(Panel B); Lane M: marker proteins; Lanes 1 and 8: *L.lactis* ATCC19435; Lanes 2 and 7: KG9408; Lanes 3 and 6: MS93003; Lanes 4 and 5: NSS9310; increase in staining intensity is seen for proteins of approx. 30 to 35 and 45 to 50 kDa in KG9408 and MS93003 compared to NSS9310 (arrowheads).

References

Alim SR, Kawai K, and Kusuda R (1996): Comparative pathogenicity study on antigenically variant strains of *Enterococcus seriolicida*. J. Fish Dis., 19:39-46

Alim SR, Hossain MA, Choedhury EK, and Kusuda R (2001): G1 antigen: a cell-surface immunoprotective 96kDa glycoprotein from the virulent fish pathogen *Enterococcus seriolicida* , its purification and characterization. Lett. Appl. Microbiol., 32: 357-361

Aoki T, Takami K, and Kitao T (1990): Drug resistance in a non-hemolytic *Streptococcus* sp. isolated from cultured yellowtail, *Seriola quinqueradiata*. Dis. Aquat. Org., 8:171-177

Barnes AC, Guyot C, Hansen BG, Mackenzie K, Horne MT, Ellis AE (2002a): Resistance to serum killing may contribute to differences in the abilities of capsulate and non-capsulated isolates of *Lactococcus garvieae* to cause disease in rainbow trout (*Oncorhynchus mykiss* L.). Fish Shellfish Immunol.,12:155-168

Barnes AC, Guyot C, Hansen BG, Horne MT, Ellis AE (2002b):Antibody increases phagocytosis and killing of *Lactococcus garvieae* by rainbow trout (*Oncorhynchus mykiss* L.) macrophages. Fish Shellfish Immunol.,12:181-186

Carson J, Gudkovs N, and Austin N (1993): Characteristics of an *Enterococcus*-like bacterium from Australia and South Africa, pathogenic for rainbow trout, *Oncorhynchus mykiss* (Walbaum). J. Fish Dis., 16:381-388

Chen SC, Lin YD, Liaw LL, and Wang PC (2001): *Lactococcus garvieae* infection in the giant freshwater prawn *Macrobranchium rosenbergii* confirmed by polymerase chain reaction and 16S rDNA sequencing. Dis. Aquat. Org.,45:45-52

Eldar A, Ghittino C, Asanta L, Bozzetta E, Goria M, Prearo M, and Bercovier H (1996): *Enterococcus seriolicida* is a junior synonym of *Lactococcus garvieae*, a causative agent of septicemia and meningoencephalitis in fish. Curr. Microbiol., 32:85-88

Eldar A, Goria M, Ghittino C, Zlotkin A, and Bercovier H (1999): Biodiversity of *Lactococcus garvieae* strains isolated from fish in Europe, Asia and Australia. Appl. Environ. Microbiol., 65:1005-1008

Handley PS, and Jacob AE (1981): Some structural and physiological properties of fimbriae of *Streptococcus faecalis*. J. Gen. Microbiol., 127: 289-293

Hirono I, Yamashita H, Park C, Yoshida T, and Aoki, T (1999): Identification of genes in a KG− phenotype of *Lactococcus garvieae*, a fish pathogenic bacterium, whose proteins react with anti-KG− rabbit serum. Microb. Pathog., 27:407-417

Iida T, Wakabayashi H, and Egusa S (1982): Vaccination for control of streptococcal disease in cultured yellowtail. Fish Pathol., 21:33-38

Kitao T (1982): The methods for detection of S*treptococcus* sp. Causative bacteria of streptococcal disease of cultured yellowtail, *Seriola quinqueradiata*, especially, their cultural, biochemical and serological properties. Fish Pathol., 17:17-26 (in Japanese with English abstract)

Kitao T (1993): Streptococcal infection. In: Inglis V, Roberts RJ, Bromage NR (eds) Bacterial diseases of fish. Blackwell Scientific Publications, Oxford, p 196-210

Kusuda R, Kawai K, Salati F, Banner CR, and Fryer JL (1991): *Enterococcus seriolicida* sp. nov., a fish pathogen. Int. J. Syst. Bacteriol., 41:406-409

Laemmli UK (1970): Cleavage of the structural proteins during the assembly of the head of bacteriophage T4. Nature,227: 680-685

Lancefield RC (1962): Current knowledge of type specific M antigens of group A Streptococci. J. Immunol., 85, 307-313

Marques LRM, Toledo MRF, Silva NP, Magalhaes M, and Trabulsi LR (1984) :Invasion of Hela cells by *Edwardsiella tarda*. Curr. Microbiol., 10: 129-132

Nakasone N, and Iwanaga M (1993): Cell-associated haemagglutinin of classical *Vibrio cholerae* O1 with reference to intestinal adhesion. FEMS Microbiol. Lett., 113: 67-70

Okada T, Minami T, Ooyama T, Yasuda H, and Yoshida T (2000): Capsular and non-capsular antigen localization on *Lactococcus garvieae* isolated from yellowtail, *Seriola quinqueradiata*. Fish. Sci., 66:992-994

Ooyama T, Kera A, Okada T, Inglis V, and Yoshida T (1999): The protective immune response of yellowtail *Seriola quinqueradiata* to the bacterial fish pathogen *Lactococcus garvieae*. Dis. Aquat. Org., 37:121-126

Park KH, Matsuoka S, Nakai T, and Muroga K (1997): A virulent bacteriophage of *Lactococcus garvieae* (formerly *Enterococcus serilocida*) isolated from yellowtail *Seriola quinqueradiata*. Dis. Aquat. Org.,

29:145-149

Park KH, Kato H, Nakai T, and Muroga K (1998): Phage typing of *Lactococcus garvieae* (formerly *Enterococcus seriolicida*) a pathogen of cultured yellowtail. Fish. Sci., 64:62-64

Paterson WD, Douglas RJ, Grinyer I, and McDermott LA (1969): Isolation and preliminary characterization of some *Aeromonas salmonicida* bacteriophages. J. Fish Res. Board Can., 26:629-632

Phillips GN Jr, Flicker PF, Cohen C, Manjula BN, and Fischetti VA (1981): Streptococcal M protein: alpha-helical coiled-coil structure and arrangement on the cell surface. Proc. Natl. Acad. Sci. USA.,78: 4689-4693

Reed LJ, and Muench H (1938): A simple method of estimating fifty percent end point. Am. J. Hyg., 9: 267-287

Roberson BS (1990): Bacterial agglutination. In: Stolen JS, Fletcher TC, Anderson DP, Roberson BS, and van Muiswinkel WB (eds) Techinques in fish immunology. SOS Publications, Fair Haven, NJ,p81-86

Sato H, Ninomiya M, Kawai K, and Kusuda R (1996): Protection and opsonic activity in the serum of yellowtail *Seriola quinqueradiata* immunized with *Enterococcus seriolicida* formalin-killed cells. Nippon Suisan Gakkaishi,62:939-940 (in Japanese with English abstract)

Schmidtke LM, and Carson J (1999): Induction, characterization and pathogenicity in rainbow trout *Oncorhynchus mykiss* (Walbaum) of *Lactococcus garvieae* L-forms. Vet. Microbiol., 69:287-300

Smith SA (1992): Affinity purification of serum immunoglobulin from fish.

In:Stolen JS, Fletcher TC, Anderson DP, Kaattari SL, and Rowley AF (eds) Techniques in fish immunology. SOS Publications, Fair Haven, NJ, p125-130

Towbin H, Staehelin T, and Gordon J (1979): Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA., 76: 4350-4354

Yoshida T, Eshima T, Wada Y, Yamada Y, Kakizaki E, Sakai M, Kitao T, and Inglis V (1996): Phenotypic variation associated with anti-phagocytic factor in the bacterial fish pathogen, *Enterococcus seriolicida*. Dis. Aquat. Org., 25:81-86

Yoshida T, Endo M, Sakai M, and Inglis V (1997): A cell capsule with possible involvement in resistance to opsonophagocytosis in *Enterococcus seriolicida* from yellowtail, *Seriola quinqueradiata*. Dis. Aquat. Org., 29:233-235

Chapter IV

Application of attenuated *Lactococcus garvieae* strain lacking a virulence-associated capsule on its cell surface as a live vaccine in yellowtail *Seriola quinqueradiata* Temminck and Schlegel

Introduction

Lactococcus garvieae is a fish pathogen that causes lactococcal infection in yellowtail *Seriola quinqueradiata*, amberjack *Seriola dumerili* (Kusuda *et al.*, 1991; Kitao, 1993) and rainbow trout *Oncorhynchus mykiss* (Schmidtke and Carson, 1999). *L. garvieae* isolated from yellowtail was divided into two phenotypes; capsulated (KG−) and non-capsulated (KG+) (Yoshida *et al.*, 1996 and 1997; Ooyama *et al.*, 1999 and 2002; Okada *et al.*, 2000). In fish, capsulated *L. garvieae* (KG−) are more virulent than non-capsulated *L. garvieae* (KG+) because the capsulated form is resistant to opsonophagocytosis (Alim *et al.*, 1996; Yoshida *et al.*, 1997). Therefore, the capsule surrounding the bacterial cell is believed to play an important role in the pathogenicity of *L. garvieae* (Barnes *et al.*, 2002a; Ooyama *et al.*, 2002). Injectable or oral vaccines have been developed recently, and the efficacy of these vaccines for the prevention of *L. garvieae* infection was demonstrated in Japan (Hirokawa *et al.*, 2004). In fish, although the precise mechanisms of protective immunity against *L. garvieae* infection remain unknown, humoral immunity in immunized fish is known to play an important role in protection against *L. garvieae* infection (Barnes *et al.*, 2002b; Ooyama *et al.*, 2002).

Previous reports (Ooyama *et al.*, 1999 and 2002; Hirokawa *et al.*, 2004) described that a formalin-killed attenuated strain (MS93003A) as well as a formalin-killed virulent strain (MS93003V) conferred immune protection against capsulated virulent strains in fish. Therefore, the aim of this study was to demonstrate the efficacy of the live *L. garvieae* MS93003A strain as a live vaccine to protect yellowtail *S. quinqueradiata* against infection by virulent *L. garvieae*.

Materials and methods

Bacterial strains.

The attenuated strain (MS93003A) was developed from the parent strain (MS93003V, capsulated strain) and subcultured on Todd-Hewitt agar medium (TH) (Difco, Detroit, MI, USA) supplemented with 0.1% of 2,3,5-triphenyltetrazolium chloride (TTC), as described by Ooyama *et al.* (1999 and 2002).

Cell morphology.

In order to produce the mimic *in vivo* conditions of the bacteria growth, the bacterial strains (MS93003V and MS93003A) were subcultured three times at 25°C for 48 h in 10 ml of filtered normal serum (0.22 μm) from yellowtail *S. quinqueradiata* (Hirokawa *et al.*, 2004). Transmission electron microscopy was used to confirm the presence of the capsule and/or fimbriae on the cell surface, in accordance with the method described by Ooyama *et al.* (2002) and Hirokawa *et al.* (2004).

Determination of LD_{50} values.

The bacterial strains (MS93003A, MS93003V and KG9408) were cultured without shaking in TH broth at 25°C for 24 h. The cells were harvested by centrifugation and resuspended in sterile saline (0.85% NaCl). Serial dilutions were prepared in saline, and viable counts were determined by plating on TH agar medium. Ten yellowtails weighing between 40 g and 60 g were intraperitoneally (IP) inoculated with 0.1 ml of each bacterial suspension. Subsequently, virulence was examined in these fish. The fish were monitored for 14 days. Virulence was expressed as lethal dose 50 (Reed and Muench, 1938).

Preparation of formalin-killed cells and attenuated live cells.

The attenuated MS93003 strain (MS93003A) was cultured in TH broth at 25°C for 24 h. The cells were harvested by centrifugation, washed twice with sterile saline, and the cell density of the suspension was adjusted to an OD of 1.0 at 620 nm. The cells were killed by formaldehyde addition (final concentration of 0.3%) and maintained at 4°C for 24h. The attenuated MS93003A strain was also cultured in TH broth, and the cells were harvested by centrifugation, resuspended in saline and the cell density adjusted to an OD of 1.0 at 620 nm. Bacterial counts (colonies forming units; CFU or cells) were determined by plating on TH agar plates.

Vaccine trial I.

Specimens of the fish *S. quinqueradiata* (approximately 100 g) were immunized by an IP injection of formalin-killed MS93003A strain cells at two

different doses of 1.0×10^6 and 1.0×10^5 cells $fish^{-1}$ (10 fish/group). The fish were also immunized by IP injections of live MS93003A strain cells at two different doses of 1.2×10^6 and 1.2×10^5 CFU $fish^{-1}$ (10 fish/group). Fourteen days after immunization, the fish were inoculated by an IP injection of MS93003V strain cells at a dose of 5.3×10^5 CFU $fish^{-1}$. Fish mortality was monitored for 21 days. The ambient water temperature throughout the experiment was maintained between 24.5°C and 25.9°C.

Vaccine trial II.

The fish (approximately 220g) were immunized by IP injections of formalin-killed MS93003A strain cells at two different doses of 1.0×10^5 and 1.0×10^4 cells $fish^{-1}$, or by live MS93003A strain cells at three different doses of 1.5×10^5, 1.5×10^4 and 1.5×10^3 CFU $fish^{-1}$. Sixteen days after immunization, the fish were inoculated by an IP injection of MS93003V strain cells at a dose of 6.3×10^6 CFU $fish^{-1}$. An ambient water temperature between 16.1°C and 17.2°C was maintained throughout this experiment.

Vaccine trial III.

S. quinqueradiata (approximately 105 g) were immunized by an IP injection of live MS93003A strain cells at a dose of 1.0×10^6 CFU $fish^{-1}$. The fish were kept in a tank (18 tons) for 295 days. The fish were transferred to an infection room for an artificial infection test after 15, 135, and 295 days of immunization. The fish were injected with MS93003V strain cells at doses of 5.2×10^5 CFU $fish^{-1}$ on 15 days, 2.6×10^5 CFU $fish^{-1}$ on 135 days and 4.6×10^5 CFU $fish^{-1}$ on 295 days.

Virulence of MS93003A after *in vivo* passages.

The fish (120-150 g) were IP injected with 2.4×10^7 CFU $fish^{-1}$ of live MS93003A strain cells. They were maintained at water temperatures of 24.5°C and 25.9°C. Bacteria isolated from the spleen of infected fish after 24-h infection, were cultured on TH agar plates for 24 h. Bacterial colonies on TH agar were suspended in saline at approximately 1.0×10^7 CFU ml^{-1}, and these were injected into other fish by an IP injection of 10^7 CFU $fish^{-1}$. Seven fish passages were carried out. Finally, the bacteria were isolated, and a pure culture was maintained in stock at −80°C. The virulence of the MS93003A strain was compared with that of the *in vivo* passaged strain with regard to the LD_{50} value of *S. quinqueradiata* (70 g) at 25°C.

Fluctuation in bacterial counts from fish after the inoculation of live MS93003A strain cells.

The fish (approximately 333g) were IP injected with live MS93003A strain cells having a cell density of 3.1×10^6 CFU $fish^{-1}$. Three fish were sampled after 1, 2, 3, 4, 5, 6, 7, 8, 11 and 19 days of injection. The number of bacterial cells in the blood, spleen, and brain was counted by the plate counting method, as described by Ooyama *et al.* (2002). These organs and the blood were aseptically sampled and homogenized or diluted with physiological saline (0.85% NaCl) supplemented with heparin (100 units ml^{-1}), and a spread plate method using TH agar plates supplemented with TTC (final concentration: 0.0015%) was used to avoid bacterial contamination from the fish surface. In particular, some bacteria such as *Vibrio* sp. swarmed on the agar plate, which disturbed the *L. garvieae* cell count. The enumeration of

CFU of *L. garvieae* was examined after a 72-h incubation period at 25°C and expressed as CFU g^{-1} for brain and spleen and CFU ml^{-1} for the blood.

Statistical analysis.

A statistical analysis of the protective efficacy of the vaccine was performed using Fisher's protected least-squares difference test.

Results

Cell morphology and virulence.

The cell morphologies of the MS93003V strain and the MS93003A strain are shown in Fig. 5. When cultured in filtered sterile serum of yellowtail, the MS93003V strain cells showed a well-developed capsule on the cell surface that has fimbriae. On the contrary, the MS93003A strain cells showed short fimbriae without a well-developed capsule. The LD_{50} values of the virulent KG9408 strain (Ooyama *et al.*,2002) and the MS93003V strain was $< 1.0 \times 10^3$ CFU $fish^{-1}$ whereas that for the MS93003A strain was $> 1.0 \times 10^6$ CFU $fish^{-1}$.

Vaccine trials I, II and III.

The efficacy of two doses (10^6 and 10^5) of the formalin-killed MS93003A strain cells and the live MS93003A strain cells as vaccines for protection against virulent *L. garvieae* infection at a relatively high water temperature (between 24.5°C and 25.9°C) is given in Table 11. Both vaccines were effective at a dose of 10^6 CFU or cells $fish^{-1}$ ($P < 0.01$). However, the formalin-killed MS93003A strain cells at doses of 10^5 cells $fish^{-1}$ were not

very effective. Although the live MS93003A vaccines were effective at doses of 1.5×10^5, 10^4, and 10^3 CFU fish^{-1}, no protection was observed with the formalin-killed MS93003A vaccines at doses of 1.0×10^5 and 10^4 cells fish^{-1} (Table 12) at lower water temperatures (between 16.1°C and 17.2°C). The live MS93003A strain cells contributed toward long-lasting protection to fish against artificial infection caused by the MS93003V strain. The *L. garvieae* cells were not found in vaccinated fish on 15 days and 135 days after the infection. The live MS93003A vaccine conferred long-lasting protection even after 295 days of immunization, and no *L. garvieae* cells were recovered from eight survivors of infected fish (Table 13).

Virulence after *in vivo* passages.

The virulence of the MS93003A strain cells was compared with that of the strain obtained after seven *in vivo* passages. Both strains caused no mortality at a dose of 1.0×10^6 CFU fish^{-1}. No morphological changes were found in the MS93003A strain after *in vivo* passages.

Fluctuation of bacterial counts in tissues.

Bacterial counts of the *L. garvieae* MS93003A cells in infected fish are shown in Table 14; these cells were recovered from the spleen until 8 days after the inoculation. Although no bacterial cells were detected from the brain, fewer counts were observed in the blood of one fish sample, after the 1st and 2nd day of inoculation of live MS93003A cells (Table 14).

Discussion

A capsulated strain of *Lactococcus garvieae* is more virulent to yellowtail *Seriola quinqueradiata* than a non-capsulated strain (Alim *et al.*, 1996; Ooyama *et al.*, 1999 and 2002). A capsule present on the cell surface is believed to be one of the virulence factors in *L. garvieae* (Yoshida *et al.*, 1996 and 1997). Hirokawa *et al.* (2004) reported that the virulent *L. garvieae* cells isolated from yellowtail had well-developed capsules, and when these cells were cultured in the presence of fish serum that fimbriae were readily found on the cell surface. Growth of some bacterial pathogens was reproduced in serum in order to identify surface components. These components were more clearly observed on the cell surface under *in vivo* growth conditions such as in serum than under *in vitro* growth conditions such as in broth or agar (Williams, 1988). This study has demonstrated the existence of peritrichous short fimbriae on the MS93003A cells cultured in serum. A previous study (Hirokawa *et al.*, 2004) reported that formalin-killed MS93003A cells cultured in the artificial medium (THB) as well as in serum conferred strong protective immunity in fish against capsulated *L. garvieae* cells. In this study, at a lower dose of cells, the live MS93003A cells conferred a stronger protective effect than formalin-killed MS93003A cells cultured in THB when yellowtails were immunized at relatively lower water temperatures (between 16.1°C and 17.2°C). Therefore, although the MS93003A cells carry protective antigens even if the cells were cultured in an artificial medium (THB), the MS93003A cells may produce more protective antigens in fish than under artificial culture conditions. Either the formalin-killed MS93003A cells or

the live MS93003A cells could confer immunity in fish without signs of pathogenicity. Therefore, the live MS93003A cells without virulence-associated capsules, which may produce more protective antigens *in vivo*, were applicable as a live vaccine.

Ooyama *et al.* (2002) reported the difficulty of differentiating fimbriae from capsules on a cell surface after the cell was treated with rabbit immune serum against the capsulated *L. garvieae* strain. This study has clearly demonstrated the existence of the components of peritrichous short fimbriae on a MS93003A strain cell that was cultured in yellowtail serum. Therefore, it is plausible that peritrichous short fimbriae are the same as the microcapsules found on MS93003A cells when the cells were treated with immune serum (Ooyama *et al.*, 2002).

Recovery of virulence in the MS93003A cells was not confirmed after *in vivo* passages in fish, and the cells did not change to the highly capsulated phenotype. Hirokawa *et al.* (2004) suggested that the well-developed capsule on the cell surface associated with virulence might not be required to confer protection against *L. garvieae* infection (Ooyama *et al.*, 2002). It is also suggested that the antigens inducing protection against the *L. garvieae* virulent cells were components of the cell-surface and not the virulence-associated capsule.

At a relatively lower water temperature, application of live MS93003A cells as a vaccine conferred a stronger immunity to fish when compared with that conferred by formalin-killed *L. garvieae* cells. *L. garvieae* infection in fish farms was observed throughout the year (Kitao 1993; Kusuda and Salati,

1999). In Japan, the injectable formalin-killed vaccines for yellowtail and amberjack against *L. garvieae* were generally recommended at a temperature higher than 20°C (May to August) to achieve protective immunity. Therefore, an attenuated live MS93003 vaccine could be applied during periods of lower temperature.

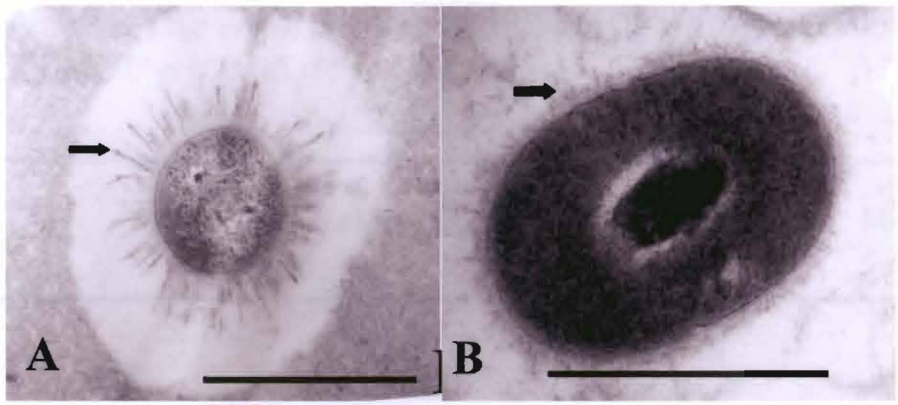

Fig. 5. Virulent (A) and attenuated (B) cells of *Lactococcus garvieae* cultured in filtered yellowtail serum. A: MS93003V (virulent) shows fimbriae (indicated by an arrow) with a well-developed capsule on its cell surface. B: MS93003A (attenuated) shows peritrichous short fimbriae (indicated by an arrow) without a well- developed capsule on its cell surface. Bars indicate 1 μ m.

Table 11. *Seriola quinqueradiata* immunized with *Lactococcus garvieae* at water temperature between 24.5°C and 25.9°C. Fish mortality (number of dead fish/number of infected fish) after immunization with two different doses of formalin-killed MS93003A and live MS93003A cells. The results were significantly different from those of non-treated control fish (*$p < 0.05$, **$p < 0.01$).

Vaccine	Dose (cells or CFU fish^{-1})	Motality
Formalin-killed MS93003A cells	1.0×10^6	0/10**
	1.0×10^5	6/10
Live MS93003A cells	1.2×10^6	0/10**
	1.2×10^5	2/10*
Non-treated control	—	9/10

Table 12. *Seriola quinqueradiata* immunized with *Lactococcus garvieae* at water temperature between 16.1°C and 17.2°C. Fish mortality (number of dead fish/number of infected fish) after immunization with different doses of formalin-killed MS93003A cells and live MS93003A cells. The results were significantly different from those of non-treated control (*$p < 0.05$, **$p < 0.01$).

Vaccine	Dose (cells or CFU $fish^{-1}$)	Motality
Formalin-killed MS93003A cells	1.0×10^5	9/10
	1.0×10^4	9/10
Live MS93003A cells	1.5×10^5	1/10**
	1.5×10^4	3/10*
	1.5×10^3	1/10**
Non-treated control	—	10/10

Table 13. *Seriola quinqueradiata* infected with *Lactococcus garvieae*. Fish mortality (number of dead fish/number of infected fish) after immunization with live MS93003A cells. The results were significantly different from those of non-treated control (*$p < 0.05$, **$p < 0.01$).

Vaccine	Days		
	15	135	295
Live MS93003A cells	0/10**	0/10**	2/10*
Non-treated control	9/10	8/10	8/10

Table 14. Fluctuation of bacterial counts of the brain, spleen and blood sampled from fish inoculated with live MS93003A cells

Days after infection	Brain (CFU g^{-1})	Spleen (CFU g^{-1})	Blood (CFU ml^{-1})
1	N.D	2.8×10^4	N.D
	N.D	7.3×10^4	1.0×10^2
	N.D	2.2×10^4	N.D
2	-	1.1×10^4	N.D
	-	8.5×10^3	1.0×10^2
	-	1.0×10^3	N.D
3	N.D	1.5×10^3	N.D
	N.D	4.8×10^3	N.D
	N.D	3.1×10^3	N.D
4	-	5.7×10^3	N.D
	-	3.8×10^2	N.D
	-	N.D	N.D
5	-	6.9×10^3	N.D
	-	2.6×10^4	N.D
	-	N.D	N.D
6	N.D	N.D	N.D
	N.D	N.D	N.D
	N.D	N.D	N.D
7	N.D	1.4×10^3	N.D
	N.D	N.D	N.D
	N.D	N.D	N.D
8	N.D	7.4×10^3	N.D
	N.D	4.5×10^3	N.D
	N.D	N.D	N.D
11	N.D	N.D	N.D
	N.D	N.D	N.D
	N.D	N.D	N.D
19	N.D	N.D	N.D
	N.D	N.D	N.D
	N.D	N.D	N.D

N.D; not detection

References

Alim SR, Kawai K, and Kusuda R (1996): Comparative pathogenicity study on antigenically variant strains of *Enterococcus seriolicida.* J. Fish Dis., 19:39-46

Barnes AC, Guyot C, Hansen BG, Mackenzie K, Horne MT, and Ellis AE (2002a): Resistance to serum killing may contribute to differences in the abilities of capsulate and non-capsulated isolates of *Lactococcus garvieae* to cause disease in rainbow trout (*Oncorhynchus mykiss* L.). Fish & Shellfish Immunol., 12:155-168

Barnes AC, Guyot C, Hansen BG, Horne MT, Ellis AE (2002b): Antibody increases phagocytosis and killing of *Lactococcus garvieae* by rainbow trout (*Oncorhynchus mykiss*, L.) macrophages. Fish & Shellfish Immunol., 12:181-186

Hirokawa Y, Ooyama T, Yasuda H, Nakamura A, Irie T, Jin D-K, and Yoshida T (2004): *Lactococcus garvieae* strains from yellowtail, *Seriola quinqueradiata* carry different lengths of fimbriae on their cell surface. Fish. Sci., 70:521-523

Kitao T (1993): Streptococcal infection. In: Bacterial Diseases of Fish. V. Inglis, R.J. Roberts, and N. R. Bromage (Eds). Blackwell Scientific Publications, Oxford, UK. pp.196-210

Kusuda R, and Salati F (1999): *Enterococcus seriolicida* and *Streptococcus iniae.* In: Fish diseases and disorders, vol. 3. P.T.K. Woo and D. W. Bruno (Eds). CABI Publishing, Wallingford, UK. pp.303-317

Kusuda R, Kawai K, Salati F, Banner CR, and Fryer JL (1991): *Enterococcus*

seriolicida sp. nov., a fish pathogen. Int. J. Syst. Bacteriol., 51:169-177

Okada T, Minami T, Ooyama T, Yasuda H, and Yoshida T (2000): Capsular and non-capsular antigen localization on *Lactococcus garvieae* isolated from yellowtail, *Seriola quinqueradiata*. Fish. Sci., 66:992-994

Ooyama T, Kera A, Okada T, Inglis V, and Yoshida T (1999): The protective immune response of yellowtail *Seriola quinqueradiata* to the bacterial fish pathogen *Lactococcus garvieae*. Dis. Aquat. Org., 37:121-126

Ooyama T, Hirokawa Y, Minami T, Yasuda H, Nakai T, Endo M, Ruangpan L, and Yoshida T (2002): Cell-surface properties of *Lactococcus garvieae* strains and their immunogenicity in the yellowtail *Seriola quinqueradiata*. Dis. Aquat. Org., 51:169-177

Reed L.J., and Muench H.(1938): A simple method of estimating fifty percent end point. Am. J. Hyg., 9:267-287

Schmidtke L.M.,and Carson J. (1999): Induction, characterization and pathogenicity in rainbow trout *Onchorhynchus mykiss* (Walbaum) of *Lactococcus garvieae* L-forms. Vet. Microbiol., 69:287-300

Williams P. (1988): Role of the cell envelope in bacterial adaptation to growth *in vivo* in infection. Biochimie,70: 987-1011

Yoshida T, Eshima T, Yamada Y, Kakizaki E, Sakai M, Kitao T, and Inglis V. (1996): Phenotypic variation associated with an anti-phagocytic factor in the bacterial fish pathogen *Enterococcus seriolicida*. Dis. Aquat. Org., 25:81-86

Yoshida T, Endo M, Sakai M, and Inglis V. (1997): A cell capsule with possible involvement in resistance to opsonophagocytosis in *Enterococcus*

seriolicida from yellowtail, *Seriola quinqueradiata*. Dis. Aquat. Org., 29:233-235

Chapter V
Discussion and Conclusion

Lactococcus garvieae infection has been inflicting severe damage on marine aquaculture industries, particularly on farms raising fish of the genus *Seriola*, including the yellowtail *S. quinqueradiata*, the king fish *S. lalandi*, and the amberjack *S. dumerili* (Kawanishi *et al.*, 2006). The lethal dose 50 (LD_{50}) of virulent *L. garvieae* was less than 10^2 in intraperitoneally infected yellowtails (Kawanishi *et al.*, 2006). This LD_{50} value supported the evidence that *L. garvieae* infection caused a serious bacterial disease in cultured yellowtails. The *L. garvieae* strains were divided into two phenotypes KG– and KG+ by using a rabbit antiserum raised against cells with a non-capsulated phenotype (KG+ phenotype cells). Kitao (1982) and Yoshida *et al.* (1996 and 1997) reported that the strain having the KG– phenotype was capsulated and displayed the non-agglutinating trait against the KG+ antiserum, while that with the KG+ phenotype was non-capsulated and displayed the agglutinating trait against the homogeneous KG+ antiserum. Virulent *L. garvieae* was capsulated, and its LD_{50} value was evaluated to be approximately $<10^2$-10^3. The non-virulent strain was non-capsulated, and its LD_{50} value was greater than 10^6 (Kawanishi *et al.*, 2006). These results suggest that the cell capsule plays an important role in *L. garvieae* infection in the yellowtail.

Recently, *L. garvieae* has been isolated from not only the genus *Seriola* but also other animals (the rainbow trout *Oncorhynchus mykiss* and terrestrial

animals) and dairy foods (Foschino *et al.*, 2006). Kawanishi *et al.* (2006) indicated that with regard to the pathogenicity to the yellowtail, bacteriophage susceptibility, and pulse-field gel electrophoresis patterns, the *L. garvieae* strains isolated from the diseased fish in Japan (yellowtail, king fish, and amberjack) could be differentiated from those isolated from other sources. Compared to the *L. garvieae* strains isolated from these three fish species in Japan, those isolated from other animals (rainbow trout, cow, pig, cat, dog, and horse) were less virulent or non-virulent to the yellowtail. Therefore, it was concluded that the *L. garvieae* strain isolated from the genus *Seriola* in Japan differed from those isolated from other animals.

Vaccines for the prevention of *L. garvieae* infection in the genus *Seriola* have been used in Japanese fish farms, and their efficacy has been demonstrated. Although the precise mechanism of protective immunity against *L. garvieae* infection in fish remains unknown, vaccines have been put to practical use in fish farms. Therefore, the mechanism of protective immunity induced by vaccinating the yellowtail against *L. garvieae* was investigated. This yielded two *L. garvieae* strains with different capsular types on their cell surfaces; one strain had a highly developed capsule and the other, a microcapsule of fimbria shape. On the other hand, neither the capsule nor the microcapsule was observed on the cell surfaces of *L. garvieae* strains isolated from other sources. Therefore, the protective immunity against the virulent *L. garvieae* strain in the yellowtail was considered to be induced by these two capsular types on the cell surfaces of these organisms. Although the *L. garvieae* strain with the microcapsule on the cell surface

was non-virulent, it could be used as a vaccine after being subjected to formalin treatment. Moreover, the highly developed capsule on the cell surface of the *L. garvieae* strain was indicated to be one of the virulence factors in the yellowtail. Further investigation revealed that the short peritrichous fimbriae on the cell surface of *L. garvieae* were similar to the microcapsules on the cell surface of attenuated MS93003A cells. These results suggested that the protective immunity in the yellowtail against infection by virulent *L. garvieae* strains was induced by antigens composed of microcapsules or fimbriae and not the virulence-associated capsules. Thus, the live attenuated and the formalin-killed vaccines of *L. garvieae* could be used to induce immunity in fish. Moreover, using a lower dose of the vaccine, a stronger protective immunity was induced by live attenuated MS93003A cells than by formalin-killed ones when the yellowtails were immunized at a relatively low water temperature. Therefore, attenuated *L. garvieae* cells could be used in fish farms throughout the year in Japan.

References

Kawanishi M, Yoshida T, Yagashiro S, Kijima M, Yagyu K, Nakai T, Murakami M, Morita H, and Suzuki S (2006): Differences between *Lactococcus garvieae* isolated from the genus *Seriola* in Japan and those isolated from other animals (trout, terrestrial animals from Europe) with regard to pathogenicity, phage susceptibility and genetic characterization. J. Appl. Microbiol., 101:496-504

Kitao T (1982): The methods for detection of *Streptococcus* sp. causative bacteria of streptococcal disease of cultured yellowtail, *Seriola quinqueradiata*, especially, their cultural, biochemical and serological properties. Fish Pathol., 17:17-26

Foschino R, Picozzi C, Borghi M, Cerliani MC, and Cresci E (2006): Investigation on the microflora of caprino Lombardo cheese from raw goat milk. Ital. J. Food Sci., 18:33-50

Yoshida T, Eshima T, Wada Y, Yamada Y, Kakizaki E, Sakai M, Kitao T, and Inglis V (1996): Phenotypic variation associated with anti-phagocytic factor in the bacterial fish pathogen *Enterococcus seriolicida*. Dis. Aquat. Org., 25:81-86

Yoshida T, Endo M, Sakai M, and Inglis V (1997): A cell capsule with possible involvement in resistance to opsonophagocytosis in *Enterococcus seriolicida* from yellowtail *Seriola quinqueradiata*. Dis. Aquat. Org., 29:233-235