Short Paper

Lactococcus garvieae strains from yellowtail Seriola quinqueradiata carry different lengths of fimbriae on their cell surface

Yusuke HIROKAWA,1 Takeshi IRIE,1 Tsuyoshi OOOYAMA,2 Hiroshi YASUDA,2 Atsushi NAKAMURA,2 Deuk-Hee JIN,3 Chaivat KITTIGUL,4 AND Terutoyo YOSHIDA1*

1Department of Fisheries, Faculty of Agriculture, Miyazaki University, Miyazaki 889-2192, 2Miyazaki Prefectural Fisheries Experimental Station, Miyazaki 889-2162, Japan, 3Faculty of Marine Bioscience and Technology, Kangnung National University, Kangnung 210-702, Korea and 4Kasetsart University, Faculty of Science, Department of Microbiology, Bangkok 10900, Thailand

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Lactococcus garvieae is a serious bacterial fish pathogen of yellowtail Seriola quinqueradiata. Lactococcus garvieae strains isolated from S. quinqueradiata have been divided into non-agglutinating (capsulated strains; KG– phenotype) and agglutinating (non-capsulated or microcapsulated strains; KG+ phenotype) phenotypes using rabbit antiserum raised against non-capsulated phenotype cells.1–7 The capsulated strain is more virulent to yellowtail than the non-capsulated phenotype strain with resistance to the opsonophagocytosis of the yellowtail phagocytic cells3,4 and serum killing.8 Therefore, a cell capsule is thought to be one of the virulence factors in L. garvieae cells from yellowtail.4–9 Recently, oral or injectable vaccines have been developed for preventing L. garvieae infection in yellowtail. Although strong immunity is induced in fish by immunization of vaccines with formalin-killed L. garvieae cells, protective mechanisms in fish by the vaccine are still under investigation.7 In a previous investigation, the cell-surface properties of strains of L. garvieae cells were examined.7 Two capsular types were found: one with a highly developed capsule and one with a microcapsule carrying fimbriae-like structures projecting from the cell surface. Although both capsular-type cells induced a strong immunity in fish, another strain had neither cell capsular nor fimbriae-like structures on its cell surface, thus providing incomplete immunity for the fish.7 However, the reason why antigens elicit strong immunity for fish is still unknown, and it is possible that cell surface components are strongly related to inducing protection in fish.7 The aim of the present investigation was to re-examine cell-surface properties of various L. garvieae strains under different culture conditions.

Here, bacterial strains were cultured in Todd-Hewitt Broth (THB; Difco, Detroit, MI, USA) or on agar (THA). Normal fish sera were collected from yellowtail (n = 30, 1250–1830 g) and pooled, filtered through 0.45 μm pore-size filter (Sartorius, Goettingen, Germany), and kept at −80°C until use. Lactococcus garvieae KG9408 (KG– phenotype), MS93003 (KG+ phenotype) and NSS9310 (KG+ phenotype) strains were used in the present investigation.6,7 Agglutinating titres of the yellowtail serum against formalin-killed KG9408, MS93003 and NSS9310 were <1 : 4, 1 : 4, and 1 : 4, respectively.

Moreover, transmission electron microscopy (TEM) was performed as described by Yoshida et al. (1997).4 Briefly, KG9408, MS93003 and NSS9310 strains were cultured in 50 mL THB and killed by the addition of a final concentration of 0.3% formaldehyde. Cells were washed three times with phosphate-buffered saline (PBS), incubated with anti-KG9408 rabbit immune serum and incu-
bated for an additional hour before staining with ruthenium red for 2 h. Bacterial cells were washed five times with PBS, embedded in 3% agarose, fixed with osmium tetroxide, washed with cacodylate buffer, and dehydrated with ethanol. Cells were also embedded in Quetol 651 (Nishin EM, Tokyo Japan). Thin sections (60 nm) were post-stained with uranyl acetate and lead acetate, and observed by TEM (Hitachi-H4800MU; Hitachi, Tokyo, Japan).

For visualizing fimbriae structures on the cell surface, *L. garvieae* strains were subcultured three times in 10 mL normal serum at 25°C for 48 h. Cells were then washed five times with PBS and fixed with formaldehyde. Samples for TEM were treated, as described above, without the treatment of anti-KG9408 rabbit immune serum incubation and ruthenium red staining.

*Lactococcus garvieae* strains (KG9408, MS93003, and NSS9310) were cultured in 10 mL THB or subcultured three times in 10 mL normal serum (100%) for 48 h at 25°C, and then they were killed by a final concentration of 0.3% formaldehyde. Succeedingly, cells were washed five times by PBS, resuspended in 10 mL PBS and agitated well before injection. Each 1 mL of formalin killed cell suspensions (cell density of formalin-killed cells was over 5.0 × 10⁸ colony forming units/mL) was injected into the fish (yellowtail, 180–250 g, n = 7 × 6). Fish were also challenged with 1 mL PBS as a control (n = 7). Fish were kept in 23–25°C sand filtered seawater. Fourteen days after immunization, the fish were challenged with KG9408 at a density of 2.1 × 10⁶ CFU/fish. Last, fish mortality was monitored for 14 days.

In general, phenotypic changes in cell surface composition and metabolism take place as a response to bacterial infection. Growth of some bacterial pathogens in serum to mimic multiplication in *in vivo* conditions has led to the identification of surface components, which are not expressed by broth or agar grown cells. In the present study, *L. garvieae* strains were screened by TEM to re-examine the cell-surface structures when the cells were cultured in the yellowtail serum. We observed that all three *L. garvieae* strains grew well in the serum. Although MS93003 and NSS9310 cells were aggregated and sedimented on the bottom of the tubes, KG9408 cells grew as well as in THB. Either the highly developed capsular strain KG9408 (Fig. 1a) or the microcapsular strain MS93003 (Fig. 1b) cells carried two distinct lengths of fimbriae-like structures (i.e. very dense peritrichous array of short fimbriae and long sparse fimbriae (Fig. 1d,e) projecting from the cell surface. Although a thin layer was seen on the cell surface of NSS9310 after it was subcultured three times in serum, fimbriae-like structures could not be observed in the present study (Fig. 1c,f). This may arise from the surface components, which were not expressed under ordinary culture conditions, but were expressed in serum cultures. Vaccine experiments using cells cultured in THB or serum cultures were examined to reveal whether components expressed on NSS9310 cells in serum cultures induce immunity in fish or not. Vaccine experiments indicated that neither THB nor serum cultured NSS9310 cells induced immunity in fish against virulent KG– phenotype cells (Table 1). These results may indicate that antigens expressed on the cell surface of NSS9310 after serum cultures.

Fig. 1 *Lactococcus garvieae*. Transmission electron microscopy (TEM) of KG9408, MS93003 and NSS9310 stains. (a–c) Cells cultured in Todd-Hewitt broth were treated with antiserum raised against capsulated KG9408 KG– phenotype cells. (d–f) Cells cultured in *Seriola quinqueradiata* normal serum. (a) KG9408 KG– phenotype cells showing well-developed cell capsule (arrowhead) with faint inside layer (arrow); (b) MS93003 KG+ phenotype cells showing microcapsule (arrowhead); (c) NSS9310 KG+ phenotype cells showing neither cell capsule nor fimbriae-like structures; (d,e) KG9408 and MS93003 cells showing sparser long fimbriae (arrowhead) with peritrichous short fimbriae (arrow); (f) NSS9310 cells showing thin surface layer, but not seen fimbriae-like strucutres after cultures in *S. quinqueradiata* serum. Bar indicates 1 μm.
could not play a role in inducing immunity in fish. Further investigations are needed to differentiate between the two types of fimbriae structures and thin layers expressed on NSS9310 after serum cultures.

Attachment to the host cell is important in initiating infection by pathogens.11 Bacterial fimbriae are thought to play a role in adherence to host cells.12 The Gram-positive bacterial pathogen Enterococcus faecalis carries thin peritrichous fimbriae on its cell surface.13 The major virulence factor of Group A streptococci is the M protein, a fibrillar surface component that protects bacteria from being ingested by phagocytic cells.14 One of the virulence factors in L. garvieae is known as a cell capsule with resistance to the opsonophagocytosis of host phagocytic cells.15 However, other virulence factors are still under investigation. Capsulated phenotype cells were more hydrophilic than non-capsulated phenotype cells.3 This may suggest that the cell capsule covers hydrophobic cell surface components, such as fimbriae structures, to be more hydrophilic. The role of fimbriae structures on L. garvieae in pathogenicity is not yet known; however, it may relate to the adherence on the host fish. Further investigation is needed to examine the role of fimbriae associated with pathogenicity in L. garvieae.

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### Table 1 Seriola quinqueradiata infected with Lactococcus garvieae KG9408

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Medium</th>
<th>Mortality</th>
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<tbody>
<tr>
<td>KG9408</td>
<td>THB</td>
<td>0/7*</td>
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<tr>
<td></td>
<td>serum</td>
<td>0/7*</td>
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<tr>
<td>MS93003</td>
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<tr>
<td>Control</td>
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Fish mortality (no. dead fish/no. infected fish) in fish immunized with formaline-killed KG9408, MS93003 and NSS9310 strains of L. garvieae cultured in Todd-Hewitt broth or yellowtail serum.

*Significantly different from control by Fisher's protected least-squares difference test (P<0.05).

THB, Todd-Hewitt Broth.

### REFERENCES


