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NOTE

A cell capsule with possible involvement in resistance to opsonophagocytosis in *Enterococcus seriolicida* isolated from yellowtail *Seriola quinqueradiata*

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ABSTRACT: A cell capsule was revealed by transmission electron microscopy on the surface of non-agglutinating (KG–), but not on agglutinating (KG+), cells of the bacterial fish pathogen *Enterococcus seriolicida*. The capsule of KG– cells may be involved in resistance to opsonophagocytosis by head kidney phagocytes of yellowtail *Seriola quinqueradiata*.

KEY WORDS: Capsule · Fish pathogen · Anti-opsonophagocytosis · *Enterococcus seriolicida* · *Seriola quinqueradiata*

Enterococcal infection of yellowtail *Seriola quinqueradiata* caused by *Enterococcus seriolicida* is a serious bacterial disease in Japan (Kitao 1993). Recently, Eldar et al. (1996) and Teixeira et al. (1996) suggested *E. seriolicida* should be re-classified into a synonym of *Lactococcus garvieae* by DNA-DNA homologies.

Enterococcus seriolicida has been divided into non-agglutinating (KG–) and agglutinating (KG+) phenotypes by anti-KG+ serum. This antigenic variation arises after subculturing fresh isolates (KG– phenotype) on Todd Hewitt agar (THA; Difco Laboratories, Detroit, USA) or on *Streptococcus* agar (KF *Streptococcus* agar; Difco) supplemented with 2,3,5-triphenyltetrazolium chloride (Kitao 1982). KG– phenotype isolates are more hydrophilic than KG+ cells, resist phagocytosis and reduce chemiluminescent responses by yellowtail head kidney phagocytes. KG+ cells elicit higher serum agglutinating titers in yellowtail than the KG– phenotype. A cell surface, putative capsular material demonstrated by scanning electron microscopy may function as an anti-phagocytic factor in *E. seriolicida* and affect its immunogenicity in yellowtail (Yoshida et al. 1996a). The previous study could not confirm the presence of a capsule in KG– cells. This study demonstrates the presence of a capsule in *E. seriolicida* KG– phenotype cells.

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Materials and methods. ***Bacteria:*** The *Enterococcus seriolicida* isolates used were obtained from diseased yellowtail in Japan (Table 1). The bacteria were maintained at –70°C in 15% (vol/vol) glycerol-Todd Hewitt broth and cultured in Todd Hewitt broth (THB) for 24 h at 25°C before use. The cell phenotype was confirmed using a rabbit antiserum against KG– or KG+ cells. KG+ cells were obtained following several passages of the KG– phenotype on THA.

Preparation of antiserum: An antiserum against KG– cells was raised as described by Yoshida et al. (1996a). An anti-KG7409(KG– cells) rabbit serum with a titer of 1:1280 (agglutinating titer) against the homologous phenotype cells was used.

Quellung test: The Quellung test (Austrian 1976) was modified using rabbit anti-KG– cell serum. Briefly, cells of both phenotypes were suspended in phosphate-buffered saline (PBS) ($OD_{660\,nm}$ = 0.6). Methylene blue (0.1 ml, 1%) was mixed with 0.1 ml cell suspension, and 20 µl aliquots were spotted on glass slides and air dried. Anti-KG– cell serum was placed on the spots which were then covered with a coverslip. Slides were examined by phase contrast microscopy (magnification ×1000). Cell capsules appeared as a halo surrounding the bacterial cell.

Confirmation of cell capsule by staining: A bacterial suspension of 10^5 colony forming units (CFU) ml^{-1} in PBS was mixed with an equal volume of Indian ink (Yoshida et al. 1996b). Capsules were also stained by Muir's method (Cowan & Steel 1965). Capsule-positive control bacteria were β-haemolytic *Streptococcus* spp. MZ9301 isolated from rainbow trout *Oncorhynchus mykiss* (Yoshida et al. 1996b).

Electron microscopy: *Enterococcus seriolicida*, KG– and KG+ (NG8206, KG7409 and MZ9501) were grown overnight in 10 ml of THB, diluted 1:100 in fresh THB and incubated for an additional 5 h at 25°C. Bacteria

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

Strain (phenotype)	Location	Antiserum		No. of subcultures	Capsule by TEM
		KG7409(–)	KG7409(+)		
NG8206 (KG–)	Nagasaki	+	–	5	+
NG8206 (KG+)	Nagasaki	+	+	10	–
KG7409 (KG–)	Kagoshima	+	–	7	+
KG7409 (KG+)	Kagoshima	+	+	20	–
MZ9501 (KG–)	Miyazaki	+	–	3	+
MZ9501 (KG+)	Miyazaki	+	+	9	–

were added to 0.3% formalin and held overnight at 4°C. The killed cells were washed 3 times with PBS and resuspended in 10 ml of KG– antiserum (1:1280) diluted 1:640 with PBS and incubated for 10 h at 4°C. Bacteria were washed 3 times with PBS and capsules were stained with 0.15% ruthenium red (Sigma, St. Louis, USA) in 2% glutaraldehyde-0.1 M cacodylate buffer, pH 7.4, for 2 h. Bacteria were washed 3 times with the same buffer, embedded in 3% agarose, washed 5 times in buffer, fixed with 2% osmium tetroxide, washed 5 times with buffer, and dehydrated with ethanol. After 5 washes with propylene oxide, the cells were embedded in Quetol 651 (Nishin EM, Tokyo, Japan). Thin sections were postfixed with uranyl acetate and lead acetate, then observed by transmission electron microscopy (Hitachi-H4800MU, Japan) at an accelerating voltage of 100 kV.

Attachment or ingestion of bacteria by head kidney cells: A phagocytosis test was performed as described by Yoshida et al. (1996a). Equal volumes of KG– or KG+ (1.0×10^6 cells ml^{-1}) and yellowtail normal pooled serum (n = 5) were mixed and incubated at 4°C for 1 h. Cells then were washed 3 times with Hanks' balanced salt solution (HBSS), and adjusted to an optical density of 0.6 at 620_{nm} in HBSS. Head kidney phagocytic cells from yellowtail with mean body weight 1250 g (n = 5) were obtained by the density gradient centrifugation method described by Braun-Nesje et al. (1982). Purified head kidney cells were adjusted to 6.0×10^6 cells ml^{-1} in HBSS and allowed to adhere to a coverglass for 2 h. Previously opsonized *Enterococcus seriolicida* KG– or KG+ cells (100 µl each) were overlayed and incubated at 25°C for 1 h, rinsed with HBSS to remove free bacteria, then fixed with methanol. The cells were visualized by Giemsa staining. The number of phagocytes containing or associated with more than 5 bacteria per 100 glass-adherent cells were examined microscopically. Adherent cells such as lymphocytes were disregarded. Statistical significance for phagocytic rate was assessed using Student's *t*-test.

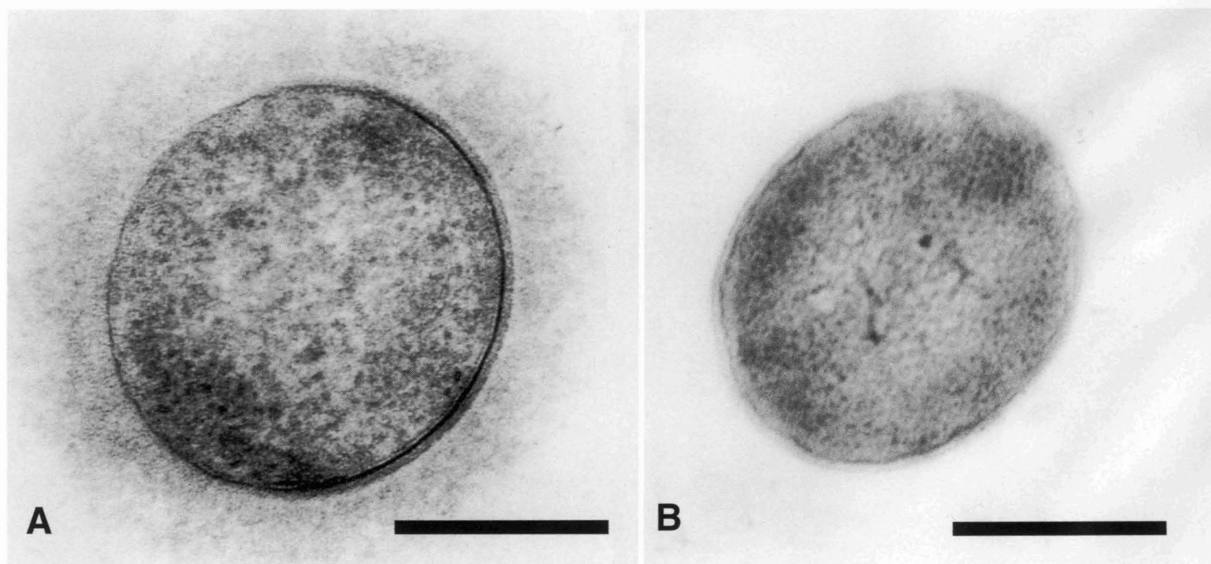

Fig. 1. Electron micrograph of *Enterococcus seriolicida* MZ9501 (KG– and KG+ phenotype). (A) Cell incubated with antiserum (KG– type) and stained with ruthenium red demonstrating an electron faint layer adjacent to the cell wall (original magnification, ×60000). (B) KG+ cell showing no layer on the cell surface (original magnification ×60000). Scale bar = 500 nm

Table 2. Phagocytic response of yellowtail *Seriola quinqueradiata* phagocytic cells against the KG– and KG+ phenotypes of *Enterococcus seriolicida*. *Value significantly lower than the value of KG+ phenotype cells ($p < 0.05$)

Strain (phenotype)	Phagocytic rate (%)
NG8206 (KG–)	10.0 ± 5.1*
NG8206 (KG+)	25.0 ± 9.4
KG7409 (KG–)	11.0 ± 3.3
KG7409 (KG+)	20.6 ± 7.8
MZ9501 (KG–)	8.0 ± 4.4*
MZ9501 (KG+)	20.2 ± 5.5

Results and discussion. No visible capsule was detected by light microscopy around *Enterococcus seriolicida* KG– or KG+ cells in all tested strains stained by Muir's, Quellung, or Indian ink. However, thin sections stabilized with antiserum and stained with ruthenium red demonstrated an electron faint layer on the surface of all KG– phenotype cells (Fig. 1A). No visible surface layer was seen on the surface of KG+ phenotype cells (Fig. 1B). A previous study (Yoshida et al. 1996a) revealed surface clumps on KG– cells by scanning electron microscopy (SEM). A surface layer was shown to be present by transmission electron microscopy in this study and could represent a cell capsule in *E. seriolicida* KG– phenotype cells.

Streptococcus pneumoniae and other Gram-positive pathogens produce exopolysaccharide capsules which contribute to virulence with resistance to phagocytosis as opposed to unencapsulated strains (Williams 1988). In earlier studies (Yoshida et al. 1996a) KG– cells were resistant to opsonophagocytosis by yellowtail head kidney phagocytes and this is supported here (Table 2). Opsonization depends on the binding of complement or antibodies at the bacterial cell surface (Arduino et al. 1994) and may be less efficient in the presence of a capsule, and therefore inversely related to the thickness of the capsule in the KG– phenotype. Kitao (1983) and Alim et al. (1996) showed KG– cells are more virulent than KG+ phenotype by means of a challenge test. The KG– factor (capsular materials) could be related to a virulence factor in *Enterococcus seriolicida*.

Enterococcus seriolicida readily underwent phenotypic conversion by subculture on artificial medium. This phenotypic conversion was dependent upon the bacterial strains as some were changed within 5 subcultures whereas other required longer subculture. Cells intermediate between KG– and KG+ cells were identified by slide agglutination using KG– and KG+ antiserum. Electron microscopy revealed the varying thickness and partial deficiency in the surface capsule, and these characteristics may be related to an intermediate phenotype. Further studies are in progress to identify the deficiency in KG– capsular materials.

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