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Comparison of antigenic proteins from *Lactococcus garvieae* KG (−) and KG (+) strains that are recognized by olive flounder (*Paralichthys olivaceus*) antibodies

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ABSTRACT

Lactococcus garvieae is an important etiological agent of lactococcosis in various fish species including olive flounder (*Paralichthys olivaceus*). In this study, proteomic and immunoproteomic analyses were employed to compare the antigenic profiles of strains KG9408, MS93003, and NSS9310 strains of *L. garvieae*. Proteomic analysis using two-dimensional gel electrophoresis (2-DE) revealed differences in five protein spots among the different *L. garvieae* strains. In immunoproteomic analysis, there was a significant difference in the 2-DE immunoblot profiles of the *L. garvieae* strains using sera collected from fish surviving infection with either *L. garvieae* strains KG9408 or NSS9310. These sera reacted with 8 and 7 unique antigenic protein spots, respectively. Heat shock protein (HSP) 70 and DNA-directed RNA polymerase were among the specific antigens recognized by the anti-NSS9310 serum. In addition, the anti-NSS9310 and anti-KG9408 olive flounder sera reacted with 25 common antigenic protein spots of all the *L. garvieae* strains, which included elongation factor (EF)-Tu, arginine deiminase (AD), inosine-5′-monophosphate dehydrogenase (IMPD), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphomannomutase (PMM), L-lactate dehydrogenase (L-LDH), 6-phosphofructokinase and UDP-galactose 4-epimerase (UDP-galactose). Based on the present results, the 8 antigens recognized by the anti-KG9408 serum and the 25 common antigens recognized by both sera may serve as potential markers for developing an effective vaccine against this bacterium.

1. Introduction

Lactococcus garvieae is an important etiological agent of lactococcosis in different fish species including yellowtail (*Seriola quinqueradiata*), rainbow trout (*Oncorhynchus mykiss*), black rockfish (*Sebastes schlegeli*), and olive flounder (*Paralichthys olivaceus*) (Kusuda et al., 1976; Chang et al., 2002; Kang et al., 2004; Baeck et al., 2006; Shin et al., 2006a). Infected fish exhibit clinical signs of hemorrhagic septicemia and meningitis (Kusuda et al., 1976; Chang et al., 2002; Vendrell et al., 2006). Serologically, isolates of *L. garvieae* are characterized into two serotypes, KG (+) and KG (−), wherein the rabbit anti-KG (−) serum agglutinates with both KG (−) and KG (+) serotypes, whereas the anti-KG (+) serum agglutinates only with the KG (+) serotype (Hirono et al., 1999; Ooyama et al., 1999, 2002). The KG (−) serotype isolates are more virulent than the KG (+) serotype due to the presence of a capsule (Okada et al., 2000; Ooyama et al., 2002), which

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renders them resistant to opsonophagocytosis and host serum killing (Yoshida et al., 1997; Barnes et al., 2002), and may also be involved in preventing an effective antibody response by masking antigenic proteins located on the cell surface (Barnes et al., 2002; Schmidtke and Carson, 2003).

Ooyama et al. (1999, 2002) characterized *L. garvieae* into three strain types. The KG9408 strain possesses a highly-developed capsule and fimbriae-like components, the MS93003 strain has a micro-capsule with fimbriae-like components projecting from their cell surface, while the NSS9301 strain has no capsule or fimbria-like structure on its cell surface. Of these strains, the MS93003 and NSS9310 strains were produced by sub-culturing the KG (−) strains on Todd–Hewitt agar (THA) supplemented with 2,3,5-triphenyltetrazolium chloride (TTC) (Hirono et al., 1999; Ooyama et al., 1999, 2002). Serologically, the KG9408 strain had the KG (−) serotype, whereas the MS93003 and NSS9310 strains had the KG (+) phenotype. Virulence testing on fish showed that the KG9408 strains were more virulent than either the MS93003 or NSS9301 strains (Ooyama et al., 2002). Several investigators have reported antigenic variation between capsulate (KG (−)) and non-capsulate (KG (+)) strains of *L. garvieae* (Hirono et al., 1999; Ooyama et al., 2002). However, these studies did not identify the antigens specific to or common between KG (−) and KG (+) strains. Recently, we reported specific and common antigens of *L. garvieae* KG (−) and KG (+) strains using immunoproteomic techniques (Shin et al., 2007a). However, there is little information on the antigenic variation between capsulated and non-capsulated strains of *L. garvieae* using serum collected from olive flounder infected with the bacteria.

In this study, we employed immunoproteomic methods to compare antigenic proteins from three different strains of *L. garvieae* (KG9408, MS93003 and NSS9310), using anti-sera from olive flounder experimentally infected with either the KG9408 or NSS9310 strains. This study may help explain the difference in virulence between the capsulate and non-capsulate stains, and may also give useful information for developing diagnostic markers and vaccine candidate proteins against *L. garvieae*.

2. Materials and methods

2.1. Preparation of bacteria

L. garvieae strains KG9408, MS93003, and NSS9310, and rabbit anti-sera for KG9408 and NSS9310 used in this study were kindly provided by Dr. Yoshida (Miyazaki University, Japan). The bacteria were stored in Todd–Hewitt broth (THB) containing 10% glycerol at −70 °C. KG9408, and MS93003 and NSS9310 were subcultured separately in 50 ml of THB, supplemented with or without 2,3,5-triphenyltetrazolium chloride (TTC) to prevent the formation of capsule at 30 °C, and grown to an OD nm of 1.0 at 610 nm for each two-dimensional gel electrophoresis (2-DE) experiment performed, according to the method of Ooyama et al. (1999). The serotypes of the cultured bacteria were confirmed by agglutination tests using rabbit serum against KG9408 and NSS9310 strains (Ooyama et al., 1999; Shin et al., 2007a). The cultured bacteria were harvested by centrifugation at 2000 × *g* for 30 min at 4 °C. The pellets were washed three times with phosphate-buffered saline (PBS; 3 mM KCl, 137 mM NaCl, 1.5 mM KH_2PO_4, and 8 mM Na_2HPO_4, pH 7.4) and resuspended in 5 ml of PBS. The bacterial suspensions (1 ml) were transferred to new tubes and centrifuged at 2000 × *g* for 10 min at 4 °C. The resulting pellets were stored at −20 °C until used for 2-DE.

2.2. Virulence tests in olive flounder

Healthy olive flounders with an average body weight and length of 150 g and 15 cm, respectively, were obtained from a commercial fish farm in Namhea County, Korea, for production of anti-sera against the KG9408 and NSS9310 strains of *L. garvieae*. Fish were divided into three groups of 30 fish each and maintained in 200 l fiber-reinforced plastic (FRP) tanks at 26–27 °C. The water was exchanged by overflowing for 2 h every day. Following anesthesia with Aqui-S®, the fish were injected intraperitoneally with 0.1 ml (10^8 CFU ml^{-1}) of the live bacteria (either strain KG9408 or NSS9310), cultured in THB. The remaining group was injected with THB broth only (the control group). All fish were maintained under identical conditions and observed for clinical signs and percentage survival for 30 days post-infection. Bacteria were isolated from dead fish and examined for hemolysis on blood agar, Gram staining, catalase and oxidase activity, and motility.

2.3. Production of anti-sera for L. garvieae strains

Anti-serum for both strains (KG9408 and NSS9310) of *L. garvieae* were used to profile antigens of the three different strains used in this study. Blood collection was performed 30 days post-infection in the challenge experiment. The fish surviving infection with KG9408 or NSS9310 were anesthetized with Aqui-S® and were then bled from the caudal vein. Serum was separated by centrifugation at 3000 × *g* for 20 min following incubation of the blood overnight at 4 °C. Sera sampled from the same group of fish were pooled and stored at −20 °C until used. Sera from fish injected with only THB were used as a negative control. Specificity of anti-sera to both strains was examined by SDS-PAGE immunoblot assays, according to the method of Shin et al. (2007b).

2.4. Extraction of bacterial proteins

Bacterial pellets were resuspended in 100 μl of sonication lysis buffer [12 mM Tris, 5% glycerol, 0.4% SDS, and 200 mM dithiothreitol (DTT)] and sonicated 8 times (XL-2020, Misonix Inc., Farmingdale, NY, USA) at 5.5 W for 30 s in an ice slurry. Cells were then disrupted by boiling for 10 min, cooled in ice, and lysed in 2-DE lysis buffer consisting of 2 M thiourea, 7 M urea, 40 mM Tris, 1% (w v^{-1}) DTT, 4% (w v^{-1}) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), and 0.5% (v v^{-1}) IPG-buffer pH 4–7. The lysates were incubated on ice for 30 min, pelleted by centrifugation at 16,000 × *g* for 30 min at 4 °C, and stored at −70 °C until used. Protein concentrations were estimated following the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as a standard.