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Environmental isolates P1SW and V3SW as a bivalent vaccine induce effective cross-protection against *Edwardsiella tarda* and *Vibrio anguillarum*

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ABSTRACT: *Edwardsiella tarda* and *Vibrio anguillarum* are severe fish pathogens. In this study, we aimed at selecting avirulent environmental isolates with application potential in the prevention of *E. tarda*- and *V. anguillarum*-associated diseases. For this purpose, we selected and analyzed 2 seawater isolates, P1SW and V3SW, belonging to the genera *Pseudomonas* and *Vibrio*, respectively. When administered to turbot *Scophthalmus maximus* via immersion and oral feeding, P1SW and V3SW at a dose of 2×10^8 colony-forming units caused no mortality, but both strains were able to disseminate into internal organs in a transient, time-dependent manner. When turbot were immunized with P1SW, V3SW, or P1SW plus V3SW (named P1V3) via immersion plus oral routes, the latter with vaccines embedded in sodium alginate microspheres, moderate protection against *E. tarda* and *V. anguillarum* was induced by V3SW, and moderate protection against *E. tarda* was induced by P1SW. Compared to P1SW and V3SW, P1V3 elicited a significantly stronger protection against both *E. tarda* and *V. anguillarum*. Immunological analysis showed that (1) P1SW, V3SW, and especially P1V3 activated head kidney macrophages, (2) P1V3 induced significantly higher levels of serum antibodies against *E. tarda* and *V. anguillarum* than P1SW and V3SW, and (3) P1V3-induced antibodies were able to bind *E. tarda* and *V. anguillarum* and enhance serum bactericidal activity. These results indicate that P1V3 as a naturally delivered vaccine elicited a humoral immune response against both *E. tarda* and *V. anguillarum* and, as a result, was cross-protective against *E. tarda* and *V. anguillarum* infection.

KEY WORDS: Bivalent vaccine · Live vaccine · Natural delivery · Cross-protection

INTRODUCTION

Edwardsiella tarda and *Vibrio anguillarum* are Gram-negative bacteria that are distributed widely in the world. They are considered to be the leading fish pathogens, with a broad host range among farmed and wild fish (Austin & Austin 2007). *V. anguillarum* is the etiological agent of vibriosis, one of the most prevalent aquaculture diseases, which affects both vertebrates and invertebrates. Vibriosis manifests itself as a hemorrhagic septicemia and can occur any time during the year, especially under stress conditions such as those caused by elevated temperature and high culture intensity. In China, vibriosis due to *V. anguillarum* infection has been documented in a number of farmed fish species, notably turbot and Japanese flounder (Fan et al. 2005).

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Edwardsiella tarda is the causative organism of edwardsiellosis, a systematic disease that has been reported to affect both marine and freshwater fish including turbot, Japanese flounder, tilapia, eels, striped bass, and catfish (Matsuyama et al. 2005, Castro et al. 2006, Joh et al. 2011, Iregui et al. 2012). In addition, *E. tarda* is a zoonotic pathogen that can be transmitted through physical contact to humans and cause gastroenteritis and other clinical disorders such as meningitis, wound infections, and septicemia (Nelson et al. 2009).

Licensed vaccines against vibriosis have been in use in Europe and America for some years and have proven to be successful in the field (Håstein et al. 2005). In China, no commercial vaccines have been developed, and, as a result, prevention of vibriosis via vaccination is a task that remains to be accomplished. The same is true for the control of edwardsiellosis, which, due to lack of efficacious vaccines, relies chiefly on the use of antimicrobial compounds. Recently, experimental vaccines against *Edwardsiella tarda* and *Vibrio anguillarum* have been reported by many research groups; however, most of these vaccines target *E. tarda* and *V. anguillarum* as individual pathogens.

Turbot *Scophthalmus maximus* is among the principal economic fish species in China. In recent years, turbot culture has been facing the problem of increased prevalence of microbial diseases, in particular those caused by bacterial pathogens such as *Edwardsiella tarda* and *Vibrio anguillarum*. The aim of this study was to develop immunoprotective approaches that can be applied in the control of *E. tarda*- and *V. anguillarum*-associated diseases in turbot. For this purpose, there are several approaches that may be employed; for example, attenuated *E. tarda* and *V. anguillarum* mutants may be developed and examined for cross-species protection as divalent vaccines. However, attenuated live vaccines are associated with the intrinsic problem of possible reversion to virulent forms. In our study, we opted to select potential vaccine candidates from avirulent environmental isolates, which have the advantage of being relatively safe and which may induce effective protection if they share common immunogens with pathogens. We screened a number of environmental isolates for strains that could induce protective immunity in turbot against *E. tarda* or *V. anguillarum*. We selected 2 isolates with such properties and examined their potentials as monovalent and divalent vaccines delivered live via the natural routes of bath immersion and oral feeding.

MATERIALS AND METHODS

Fish

Turbot (average 14.2 ± 0.5 g SD) were purchased from a local fish farm and acclimatized in the laboratory for 2 wk before experimental manipulation. Fish were fed daily with commercial dry pellets and maintained at ~18°C in aerated seawater. Before the experiment, fish were randomly sampled for the examination of bacterial recovery from blood, liver, kidney, and spleen; no bacteria could be detected from any of the examined tissues of the sampled fish. In addition, enzyme-linked immunosorbent assay (ELISA) analysis showed that the randomly selected fish were negative for serum antibodies against *Edwardsiella tarda* and *Vibrio anguillarum*. For tissue examinations, fish were euthanized with an overdose of tricaine methanesulfonate (Sigma) as described previously (Wang et al. 2009).

Bacterial strains and their characterization

Edwardsiella tarda TX1 and *Vibrio anguillarum* C312 are fish pathogens that have been reported previously (Zhang et al. 2008, Zheng et al. 2010). Both strains were cultured in Luria-Bertani broth (LB) medium at 28°C. P1SW and V3SW, 2 isolates belonging to the genera *Pseudomonas* and *Vibrio*, respectively, were isolated from seawater in the coastal region of Qingdao, China, and cultured in LB medium at 28°C. 16S rRNA-based genetic identification was performed using primers 8F and 1492R as reported previously (Zhang & Sun 2007). The 16S rRNA sequences of P1SW and V3SW have been deposited in GenBank under accession numbers JX569763 and JX569764, respectively. P1SW and V3SW were analyzed for the potential existence of plasmids using the procedure of Kado & Liu (1981).

Bacterial recovery from fish tissues

Turbot were infected with P1SW and V3SW via immersion plus oral feeding as described in the vaccination procedure (see below), except that the dosage was doubled: the oral feeding was conducted at a dose of ~0.3 g feed d^{-1} and immersion was performed in seawater containing 2×10^8 colony-forming units (CFU) ml^{-1} of bacterial cells.

Blood, liver, spleen, kidney, and gut were taken from the fish at 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 18, and 20 d after infection (5 fish per time point). The tissues were homogenized in phosphate-buffered saline (PBS). After serial dilution, the homogenates and blood were plated in triplicate on LB agar plates supplemented with 50 µg ml^{-1} ampicillin (selection marker of P1SW and V3SW). After incubation at 28°C for 48 h, the colonies that appeared on the plates were enumerated. The genetic nature of the colonies was verified by 16S rRNA sequence analysis as described above.

Preparation of vaccine-containing microspheres

To prepare vaccine-containing microspheres, P1SW and V3SW were cultured in LB medium to an optical density at 600 nm (OD_{600}) of 1. The cells were harvested by centrifugation, washed 3 times with PBS, and resuspended in PBS. To prepare P1V3 suspension, P1SW and V3SW were mixed at an equal volume. Forty milliliters of 3% (m/v) sodium alginate were mixed with 24 ml of P1SW (2.5×10^9 CFU ml^{-1}), V3SW (2.5×10^9 CFU ml^{-1}), P1V3 (1.25×10^9 CFU ml^{-1} P1SW and 1.25×10^9 CFU ml^{-1} V3SW), or PBS (control), and the mixture was emulsified by adding 1600 ml of paraffin and 8 ml of Span-80. While stirring, 40 ml of 0.15 M $CaCl_2$ were added to the emulsion, and microspheres were collected by centrifugation at 1000 × *g* (10 min). Marine fish feed (100 g; purchased from Shandong Sheng-suo Fish Feed Research Center, Shandong, China) was mixed with vaccine-containing microspheres or the control microspheres. The feed was cut into small pieces of the size approximating that of the purchased fish feed. After drying at 30°C, the feed was stored at 4°C and used within 2 d.

Vaccination

P1SW and V3SW were cultured in LB medium to OD_{600} of 0.8. The cells were harvested by centrifugation, washed 3 times with PBS, and resuspended in PBS to 2×10^9 CFU ml^{-1}. Fish described above were randomly divided into 4 groups (N = 100 per group): A, B, C, and D. Group A was immersed in seawater containing 1×10^8 CFU ml^{-1} P1SW, Group B was immersed in seawater containing 1×10^8 CFU ml^{-1} V3SW, Group C was immersed in seawater containing a combination of P1SW and V3SW (0.5×10^8 CFU ml^{-1} each), and Group D was similarly immersed in PBS-containing seawater. The immersion lasted for 4 h. The fish were then moved to tanks containing fresh seawater and subjected immediately to oral vaccination as follows. Groups A, B, and C were fed for 3 d with feed containing P1SW, V3SW, and P1SW plus V3SW microspheres (~0.15 g d^{-1}), respectively; Group D was similarly fed with feed containing control microspheres. The fish were then reared under normal conditions as described above. At 1 mo post vaccination, 34 fish were taken from each group and challenged via intraperitoneal (i.p.) injection with *Edwardsiella tarda* TX1; likewise, 34 fish were taken from each group and challenged via i.p. injection with *Vibrio anguillarum* C312. After each challenge, the fish were monitored for mortality for 3 wk, and dying fish were randomly selected for the examination of bacterial recovery from liver, kidney, and spleen as described previously (Zhang et al. 2008). Relative percent of survival (RPS) was calculated according to the following formula: RPS = [1 − (% mortality in vaccinated fish/% mortality in control fish)] × 100 (Amend 1981).

Respiratory burst activity

Turbot head kidney macrophages were prepared as reported previously (Hu et al. 2010). The cells were resuspended in L-15 medium (Thermo Scientific HyClone) and maintained in 96-well tissue culture plates. Respiratory burst activity was determined based on the method of Chung & Secombes (1988). In brief, 100 µl of 1 mg ml^{-1} nitroblue tetrazolium (Sangon) in L-15 were added to 2×10^5 macrophages in a 96-well microplate. After incubation at 25°C for 2 h, the reaction was stopped by adding 100% methanol. The plate was washed with 70% methanol, and the reduced formazan was solubilized in 100 µl of 2 M KOH and 120 µl of dimethyl sulfoxide. The plate was read at 630 nm with a microplate reader.

ELISA

Sera were collected from unvaccinated and vaccinated fish (5 at each time point) at 1 mo post vaccination. Sera were diluted 32-fold in PBST (0.1% Tween-20 in PBS) containing 1% bovine serum albumin. Serum antibodies against *Edwardsiella tarda* and *Vibrio anguillarum* were determined by ELISA as described previously (Sun et al. 2011).

Identification of potential cross-protective antigens

Outer membrane proteins (OMPs) were prepared from *Edwardsiella tarda* as reported previously (Sun et al. 2012). The proteins were then subjected to Western blot analysis as reported previously (Sun et al. 2012) with serum from P1V3-vaccinated fish. One of the proteins detected by anti-P1V3 antibodies was picked from the gel and used for mass spectrometric analysis by BGI (Shenzhen).

Serum bactericidal activity

The serum bactericidal assay was performed as follows. *Edwardsiella tarda* and *Vibrio anguillarum* were cultured to OD_{600} of 0.8 and resuspended in PBS to 1×10^6 CFU ml^{-1}. Ten microliters of bacterial suspension were mixed with 50 µl serum or PBS. The mixture was incubated at 30°C for 1 h and, after diluting in LB medium, plated onto LB agar plates. The plates were incubated at 30°C for 48 h, and the colonies that appeared on the plates were counted. The genetic nature of the colonies was verified by PCR analysis using primers specific to *E. tarda* TX1 and *V. anguillarum* C312, and the PCR products were randomly selected for DNA sequencing.

Statistical analysis

All statistical analyses were performed with the SPSS 17.0 package. A chi-squared test with Yates' correction was used for mortality analysis, and analysis of variance (ANOVA) was used for all other analyses. In all cases, the significance level was defined as $p < 0.05$.

RESULTS

Characterization of P1SW and V3SW

In an effort to select environmental isolates with potential protective effects against *Edwardsiella tarda* and *Vibrio anguillarum* infection, we conducted a preliminary experiment to examine the immune effect of a number of avirulent strains isolated from seawater. We found that strains P1SW and V3SW induced apparent protection in turbot. The 2 strains were therefore selected for further study. 16S rRNA sequence analysis showed that P1SW shares 100% identity with several strains of *Pseudomonas* sp. (GenBank accession nos. JX484 804.1, JQ396636.1, AB680968.1, and HQ224635.1), while V3SW shares 100% identity with several strains of *Vibrio* sp. (GenBank accession nos. JF836 167.1, AB498798.1, FM878645.1, and AF064637). Since some marine isolates are known to contain large, transmissible plasmids, P1SW and V3SW were examined for potential existence of plasmids using the well-tested method of Kado & Liu (1981). No plasmid was detected in P1SW and V3SW. Virulence analysis indicated that when turbot were infected via i.p. injection with 5×10^7 CFU of P1SW or V3SW, no mortality or disease occurred. When infection was performed via immersion plus oral feeding, 2×10^8 CFU of P1SW and V3SW induced no mortality, clinical symptom, or growth defect in the target fish within the observed period (40 d). However, following immersion and oral feeding, both P1SW and V3SW were detected in blood, spleen, kidney, gut, and liver in a time-dependent manner (Fig. 1). For P1SW, bacterial recoveries in all tissues were highest at 1 d post infection and then declined with time; no bacteria were detected in blood, spleen, kidney, gut, and liver by 5, 7, 7, 8, and 8 d post infection, respectively. Similar patterns of bacterial recovery were observed with V3SW.

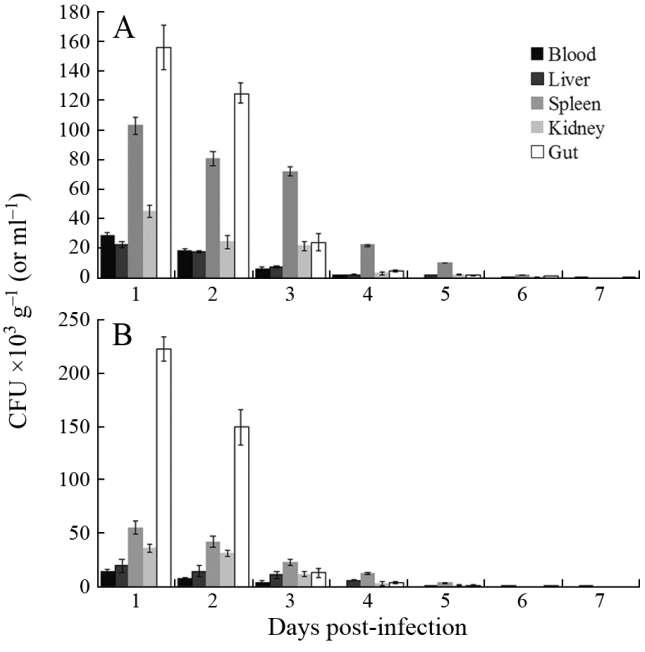

Fig. 1. *Scophthalmus maximus.* Distribution of (A) P1SW and (B) V3SW in turbot tissues following immersion and oral feeding. Liver, spleen, kidney, blood, and gut were taken at different time points and determined for bacterial recovery. Data are means ± SE (N = 5 fish $assay^{-1}$)

Immunoprotective effect of P1SW and V3SW as monovalent and divalent vaccines

Since P1SW and V3SW were capable of transient tissue dissemination following immersion plus oral administration, we examined their potentials as monovalent and divalent vaccines delivered via natural infection. For this purpose, turbot were immunized with P1SW, V3SW, P1SW plus V3SW (named P1V3), or PBS via bath immersion plus oral feeding. Fish were challenged at 1 mo post vaccination with *Edwardsiella tarda* or *Vibrio anguillarum* and monitored for mortality. The results showed that following *E. tarda* challenge, the accumulated mortalities of the fish vaccinated with P1SW, V3SW, P1V3, and PBS were 55.9, 50, 20.6, and 97.1%, respectively. Based on these results, the protection rates, in terms of RPS, of P1SW, V3SW, and P1V3 were calculated to be 42.4, 48.5, and 78.8%, respectively, with PBS as a control. Following *V. anguillarum* challenge, the accumulated mortalities of the fish vaccinated with P1SW, V3SW, P1V3, and PBS were 88.2, 38.2, 5.9, and 91.2%, respectively, which correspond to RPS rates of 3.3, 58.1, and 93.5% for P1SW, V3SW, and P1V3, respectively. The RPS rates of P1V3 against both *E. tarda* and *V. anguillarum* (i.e. 78.8 and 93.5%, respectively) were significantly ($p < 0.05$) higher than those of P1SW and V3SW. Microbiological analysis showed that *E. tarda* and *V. anguillarum* were the only bacteria recovered from the liver, spleen, and kidney of moribund fish challenged with *E. tarda* and *V. anguillarum*, respectively, suggesting that mortality was caused by the challenging organisms.

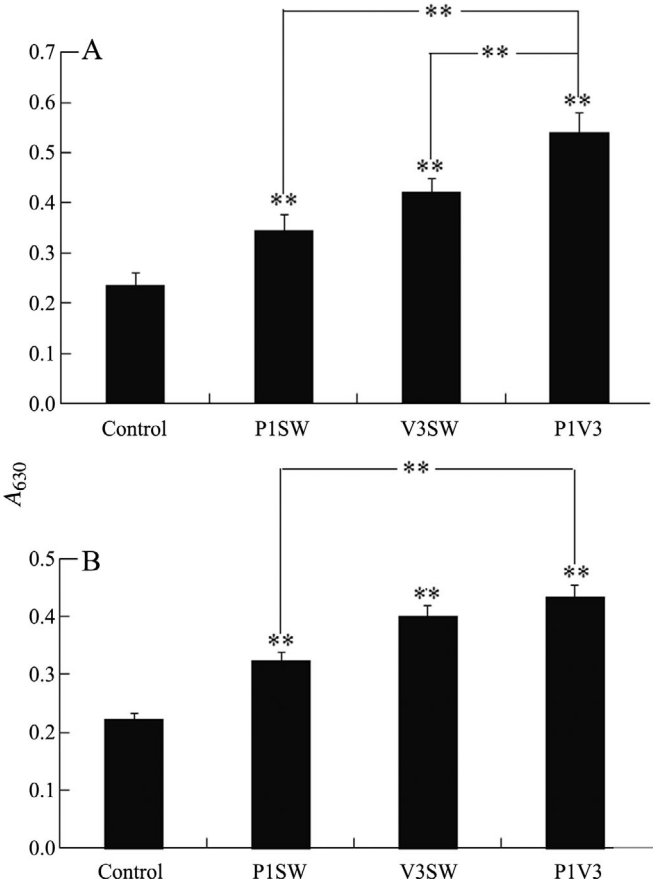

Fig. 2. *Scophthalmus maximus.* Effect of vaccination on macrophage respiratory burst (absorbance at 630 nm, A_{630}). Head kidney macrophages were taken from fish vaccinated with P1SW, V3SW, P1V3, and PBS (control) at (A) 1 d and (B) 3 d post vaccination, and respiratory burst activity of the cells was determined. Data are means ± SE (N = 5). **$p < 0.01$

Immune response induced by the vaccines

Macrophage activation

To examine whether vaccination had any effect on macrophage activity, head kidney macrophages were taken from fish vaccinated with P1SW, V3SW, P1V3, and PBS (control) at 1 and 3 d post-vaccination and examined for reactive oxygen species (ROS) production by measuring the respiratory burst activity. The results showed that compared to macrophages from control fish, macrophages from fish vaccinated with P1SW, V3SW, and P1V3 exhibited significantly higher respiratory burst activities (Fig. 2). At both examined time points, the ROS levels of P1V3-vaccinated fish were significantly higher than those of P1SW- and V3SW-vaccinated fish.

Production of serum antibodies

Production of serum antibodies in vaccinated fish was determined by ELISA. The results showed that P1SW-vaccinated fish produced antibodies against *Edwardsiella tarda* but not against *Vibrio anguillarum*, while V3SW- and P1V3-vaccinated fish produced antibodies against both *V. anguillarum* and *E. tarda* (Fig. 3). However, the levels of both anti-*E. tarda* and anti-*V. anguillarum* antibodies in P1V3-vaccinated fish were significantly higher than those in P1SW- and V3SW-vaccinated fish.

Binding of serum antibodies to bacterial cells

To examine whether vaccine-induced antibodies could interact with *Edwardsiella tarda* and *Vibrio*

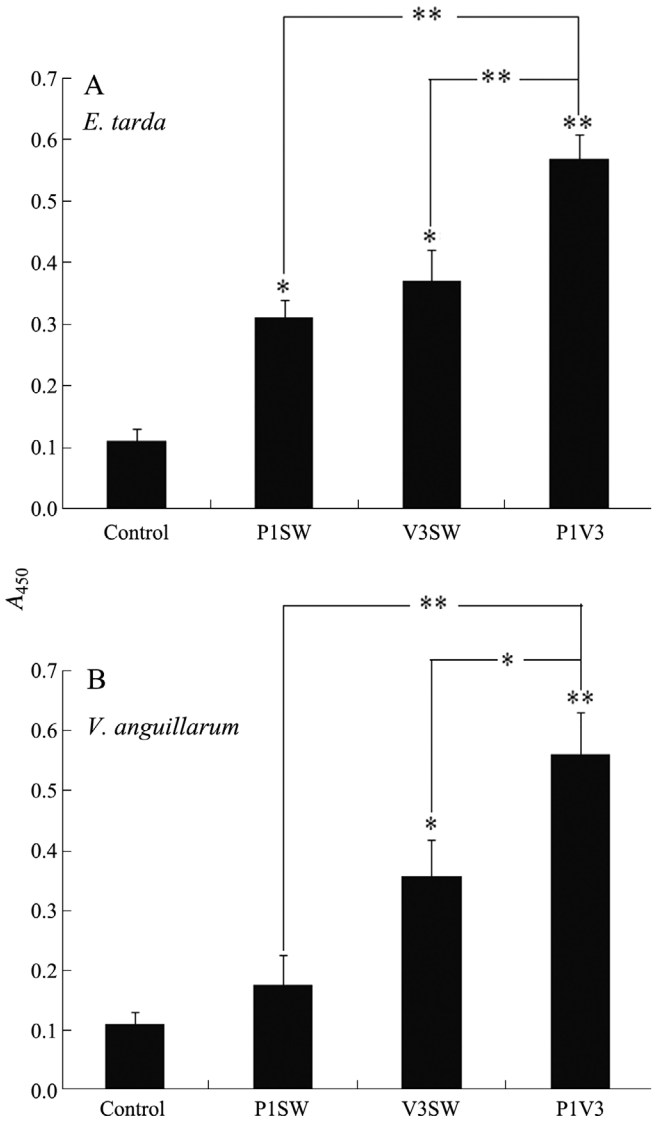

Fig. 3. *Scophthalmus maximus.* Serum antibody production in vaccinated fish. Sera were collected from fish vaccinated with P1SW, V3SW, P1V3, and PBS (control) at 1 mo post vaccination and diluted 32 times. Serum antibodies against (A) *Edwardsiella tarda* and (B) *Vibrio anguillarum* were determined by ELISA. Data are absorbance at 450 nm, shown as means ± SE (N = 5 fish assay^{-1}). *p < 0.05, **p < 0.01

anguillarum, the bacterial cells were incubated with serum from P1V3-vaccinated fish or control fish (i.e. PBS-vaccinated fish), and the bound antibodies were detected by FITC-labeled secondary antibody. Subsequent immunofluorescence microscopy showed that fluorescence was observed on *E. tarda* and *V. anguillarum* treated with serum from P1V3-vaccinated fish but not on bacterial cells treated with serum from the control fish (Fig. 4 and data not shown).

Identification of potential cross-protective antigens in *Edwardsiella tarda*

Given the above results, i.e. that P1V3-induced antibodies could bind to *Edwardsiella tarda* and *Vibrio anguillarum*, which suggested presence of common surface antigens between P1V3 and the 2 pathogens, we further examined the OMPs of *E. tarda* for potential existence of antibody-interacting antigens. The results showed that several *E. tarda* OMPs, which ranged between 20 and 85 kDa in molecular mass, were recognized by P1V3-induced antibodies (Fig. 5). One of these proteins (indicated by the arrow in Fig. 5) was subjected to mass spectrometry and was identified to be OMP-A.

Serum bactericidal activity

To examine the effect of vaccine-induced antibodies on serum bactericidal activity, sera from fish vaccinated with P1SW, V3SW, P1V3, and PBS were incubated with *Edwardsiella tarda* or *Vibrio anguillarum*, and bacterial survival rate was subsequently determined. The results showed that for *V. anguillarum*, the survival rate after treatment with the serum from P1SW-vaccinated fish was comparable to that of the control, while the survival rates were significantly reduced after treatment with sera from V3SW- or P1V3-vaccinated fish (Fig. 6). For *E. tarda*, treatment with the sera from P1SW-, V3SW-, and P1V3-vaccinated fish all significantly reduced the survival rate.

DISCUSSION

In this study, we analyzed the immunoprotective potential of 2 environmental isolates, P1SW and V3SW, as monovalent and divalent vaccines. P1SW and V3SW were isolated from seawater, and sequence analysis showed that they shared 100% identities with strains of *Pseudomonas* and *Vibrio*, respectively, suggesting that P1SW and V3SW belong to the genera of *Pseudomonas* and *Vibrio*, respectively. It is known that some marine isolates contain large, movable plasmids that can be transmitted between strains of the same and different genera (Sun et al. 2009). In our study, no plasmid was detected in P1SW and V3SW, suggesting that these 2 strains lack at least the plasmids that are detectable via the commonly used method. When turbot were infected with P1SW and V3SW at a dose of 2×10^8 CFU via immersion plus

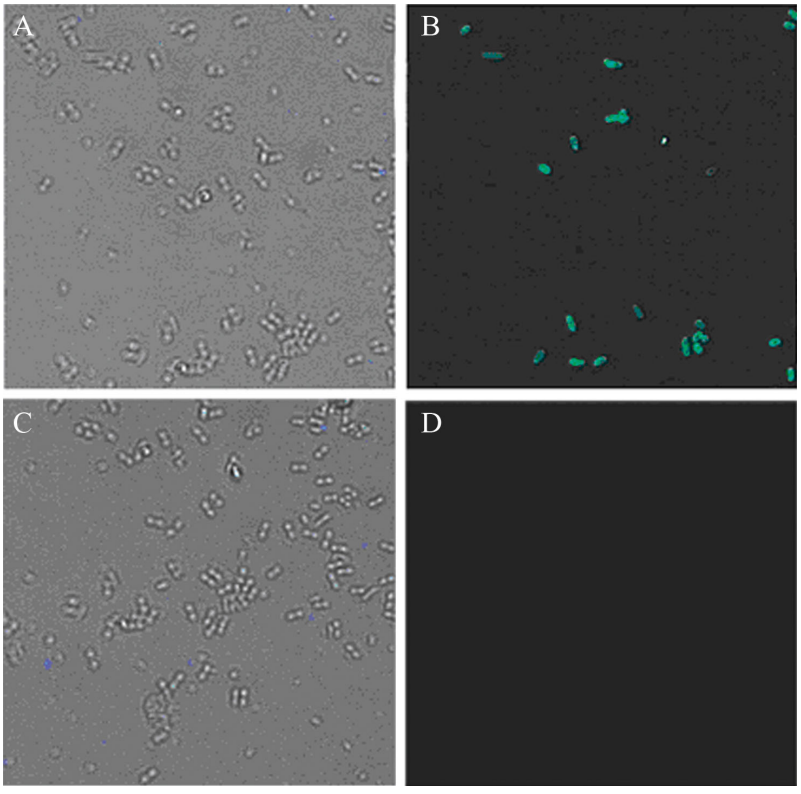

Fig. 4. *Edwardsiella tarda.* Interaction between vaccine-induced antibodies and bacterial cells. *E. tarda* was incubated with serum from (A,B) P1V3-vaccinated fish or (C,D) control fish, followed by treatment with FITC-labeled secondary antibody. Cells were observed under a microscope with (B,D) or without (A,C) fluorescence

oral feeding, no disease symptom was observed in the fish, suggesting that P1SW and V3SW are relatively avirulent. The observation that both strains were recovered from the internal organs at 5 to 8 d after infection indicates that, although P1SW and V3SW were unable to cause a successful infection leading to clinical symptoms, they possess a certain infectivity that enables the bacteria to invade into fish tissues in a transient manner.

Compared to injection vaccines, vaccines delivered via natural routes such as oral feeding and bath immersion are more advantageous in the sense that the latter are of lower cost, easier to perform, and less limited by the age of the animals (Sommerset et al. 2005). In this study, since P1SW and V3SW were capable of limited tissue dissemination, we examined their potentials as immersion and oral vaccines. Our results showed that following immersion plus oral vaccination, P1SW-vaccinated fish exhibited RPS rates of 42.4 and 3.3 % against *Edwardsiella tarda* and *Vibrio anguillarum*, respectively, while V3SW-vaccinated fish exhibited RPS rates of 48.5 and 58.1 % against *E. tarda* and *V. anguillarum*, respectively. These results indicate that as monovalent vaccines, P1SW induced moderate protection against

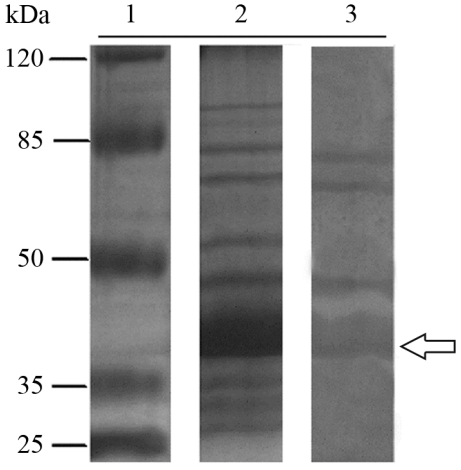

Fig. 5. *Edwardsiella tarda.* Identification of outer membrane proteins (OMPs) that reacted with P1V3-induced antibodies. *E. tarda* OMPs were subjected to SDS-PAGE analysis (Lane 2), and the proteins were then blotted with P1V3-induced antibodies (Lane 3). Arrow indicates the protein used for mass spectrometry. Lane 1: protein size marker

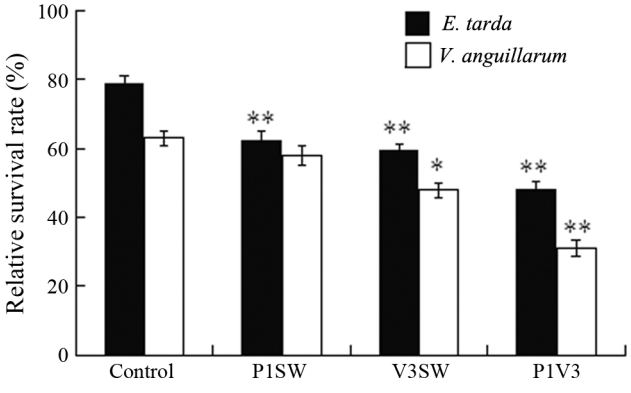

Fig. 6. *Scophthalmus maximus.* Serum bactericidal activity. Sera were collected from fish vaccinated with P1SW, V3SW, P1V3, and PBS (control), and bactericidal activity against *Edwardsiella tarda* and *Vibrio anguillarum* was determined. Data are means ± SE (N = 5), and significances between vaccinated fish and control fish are indicated with asterisks. $^{*}p < 0.05$, $^{**}p < 0.01$

E. tarda, while V3SW induced moderate protection against both *E. tarda* and *V. anguillarum*. Compared to the monovalent vaccines, the divalent P1V3 induced significantly higher levels of RPS rates against both *E. tarda* and *V. anguillarum*. The augmented protection of P1V3 against *E. tarda* is possibly due to the combined effect of P1SW and V3SW, both of which were individually protective against *E. tarda*. Since P1SW as a monovalent vaccine elicited no protection against *V. anguillarum*, the significantly increased protection of P1V3 against *V. anguillarum* suggests the possibility that in the divalent vaccine, P1SW likely stimulated the immune response of V3SW to the extent that resulted in enhanced resistance against *V. anguillarum* infection. This hypothesis is in line with the results of macrophage activation analysis and, in particular, the ELISA analysis, the latter showing that compared to fish vaccinated with the monovalent vaccines, fish vaccinated with P1V3 exhibited not only a significantly higher level of anti-*E. tarda* antibodies but also a significantly higher level of anti-*V. anguillarum* antibodies, which suggests an adjuvant-like effect of P1SW on the B cell immune response of V3SW. In addition, the protective difference observed between monovalent and divalent vaccines may also be due to differences in *in vivo* dissemination of the vaccine strains. It is possible that P1SW and V3SW in the divalent vaccine, though in a dose that was half of that in monovalent vaccines, may have a more disseminated distribution and/or longer persistence in the vaccinated fish, thus evoking stronger systemic immune response.

Cross-protection due to the presence of natural multivalent antigens in fish isolates of different genera and species has been reported previously (Swain et al. 2003, Xu et al. 2005, Liu et al. 2007, Li et al. 2010, Khushiramani et al. 2012). For example, the glyceraldehyde-3-phosphate dehydrogenase of *Edwardsiella tarda* protects Japanese flounder against *Vibrio anguillarum* (Liu et al. 2007), and the *V. parahaemolyticus* OMPs VP1061 and VP2850 induce cross-protective immune reactions against infection of *V. alginolyticus*, *Aeromonas hydrophila*, and *Pseudomonas fluorescens* (Li et al. 2010). In addition, protections against multiple bacterial pathogens are known to be conferred by some probiotics, which stimulate innate and adaptive immune response (Pirarat et al. 2006, Nayak 2010). In our study, consistent with the protection results, ELISA analysis showed that anti-*E. tarda* antibodies were detected in fish vaccinated with all 3 vaccines, whereas anti-*V. anguillarum* antibodies were detected in fish vaccinated with V3SW and P1V3 but not in fish vaccinated with P1SW, suggesting that the immunoprotective effects of the vaccines were at least in part due to induction of protective immunity against *E. tarda* (for P1SW) or against both *E. tarda* and *V. anguillarum* (for V3SW and P1V3). This conclusion is supported by the observations that P1V3-induced antibodies bound *E. tarda* and *V. anguillarum*, and that one of the antibody-interacting OMPs of *E. tarda* was identified to be OMP-A, which is a conserved bacterial protein known to possess vaccine potential (Wang et al. 2012). These results suggest the presence of common surface antigens between P1SW and *E. tarda* and between V3SW and *E. tarda* and *V. anguillarum*. In agreement with these observations, P1SW-induced antibodies enhanced serum bactericidal activity against *E. tarda* but not *V. anguillarum*, while V3SW- and P1V3-induced antibodies enhanced serum bactericidal activity against both *E. tarda* and *V. anguillarum*.

In conclusion, the results of this study indicate that P1SW and V3SW as monovalent vaccines induce moderate protective immunity against *Edwardsiella tarda* and/or *Vibrio anguillarum*, due most likely to the existence of shared antigenicity between P1SW and V3SW and the 2 pathogens. Compared to monovalent P1SW and V3SW, the divalent vaccine P1V3 induces significantly elevated and effective cross-protection against both *E. tarda* and *V. anguillarum*. Since the vaccination was conducted via bath immersion and oral feeding, which is of low cost, P1V3 has a good potential for aquaculture application.

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