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C312M: an attenuated *Vibrio anguillarum* strain that induces immunoprotection as an oral and immersion vaccine

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ABSTRACT: *Vibrio anguillarum*, a Gram-negative bacterial pathogen, is the causative agent of vibriosis that affects a wide range of aquatic animals. In this study, we obtained a mutant *V. anguillarum*, C312M, derived from the pathogenic *V. anguillarum* C312 by selection of rifampicin resistance. C312M was slower in growth than the wild type C312, particularly under conditions of iron depletion. Compared to C312, C312M was altered in protein production profile and exhibited a dramatically increased median lethal dose. Safety analysis showed that C312M was stable in virulence in the absence of selective pressure. To examine the potential of C312M as a live attenuated vaccine, Japanese flounder *Paralichthys olivaceus* were vaccinated with C312M via oral, immersion, and oral plus immersion routes. Microbiological analysis showed that C312M was recovered from the gut, liver, kidney, and spleen of the vaccinated fish in 1 to 14 d post-vaccination. When the fish were challenged with C312 at 1 mo post-vaccination, C312M-vaccinated fish exhibited relative percent survival rates of 60 to 84%. Comparable protection was observed when the fish were challenged with a heterologous *V. anguillarum* strain. Further analysis showed that C312M-vaccinated fish produced specific serum antibodies which enhanced serum bactericidal activity in a manner that is probably complement-dependent. These results indicate that C312M is highly attenuated in virulence but still retains residual infectivity, and that C312M is an effective vaccine when delivered alive via immersion and oral feeding.

KEY WORDS: *Vibrio anguillarum* · Attenuated vaccine · Rifampicin resistance · Immersion · Oral feeding

INTRODUCTION

Vibriosis is a hemorrhagic septicemia that affects cultured and wild marine fish (Frans et al. 2011). The causative agents of vibriosis are several bacterial species belonging to the genus *Vibrio*, in particular *V. anguillarum*, a Gram-negative rod-shaped bacterium with polar flagellum, which infects approximately 50 different fish species (Actis et al. 2011). To date, 23 different serotypes of *V. anguillarum* have been identified, of which serotypes O1 and O2 are distributed worldwide and are most closely associated with vibriosis in fish (Norqvist et al. 1990, Toranzo & Barja 1990, Milton et al. 1992, Larsen et al. 1994). In China, *V. anguillarum* is ranked among the most prevalent aquatic pathogens and causes disease in both invertebrates and vertebrates, the latter including various fish species such as turbot, Japanese flounder, and eel (Zhang et al. 2004).

Antibiotics, notably rifampicin and novobiocin, have been used in the selection of bacterial mutants

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with attenuated virulence based on the observation that acquisition of antibiotic resistance is sometimes accompanied by significant loss of pathogenicity (Bhatnagar et al. 1994, Campbell et al. 2001). Rifampicin is a bactericidal that kills bacteria by inhibiting the process of transcription, thereby disabling protein expression required for normal bacterial growth and survival. Rifampicin works by binding tightly to the RNA polymerase and blocking exit of the newly synthesized RNA transcript, which stops the transcription process. Although the precise mechanism of virulence reduction in rifampicin-resistant mutants is not clear, several dramatically attenuated fish bacterial pathogens, such as *Edwardsiella ictaluri*, *E. tarda*, and *Flavobacterium columnare*, have been selected with rifampicin and found to possess vaccine potentials (LaFrentz et al. 2008, Sun et al. 2010, Pridgeon & Klesius 2011a,b).

In this study, a mutant *Vibrio anguillarum* strain, C312M, was obtained by repeated selection of rifampicin-resistant derivatives of *V. anguillarum* C312, a pathogenic strain isolated from diseased flounder. We examined the virulence of C312M and determined in a flounder model the potential of C312M as a live vaccine delivered via immersion and oral routes. We found that C312M was able to elicit effective immunoprotection against not only C312 but also a heterogeneous *V. anguillarum* strain that differs from C312 in serotype.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Vibrio anguillarum C312 (serotype O1) is a pathogenic strain isolated from diseased flounder (Zheng et al. 2010). *V. anguillarum* 19264 (serotype O2) was purchased from the American Type Culture Collection. Both strains were cultured in Luria-Bertani broth (LB) medium at 28°C. Where indicated, 2,2′-dipyridyl was added to the culture medium at a final concentration of 200 µM. C312 shows natural resistance against ampicillin but is sensitive to chloramphenicol, tetracycline, and rifampicin. C312 was analyzed for potential existence of plasmid using the procedure of Kado & Liu (1981), and the results were negative. C312M was obtained as follows. C312 was cultured in LB medium to an OD_{600} (optical density at 600 nm) of 0.9, and 100 µl of the culture was plated on a LB agar plate containing 1.5 µg ml^{-1} rifampicin (Sangon). The plate was incubated at 28°C for 4 d. One of the colonies that emerged on the plate was randomly selected from the plate and cultured in LB medium containing 3 µg ml^{-1} rifampicin to an OD_{600} of 0.8. One hundred microliters of the culture was removed and sub-cultured in LB medium containing 5 µg ml^{-1} rifampicin. The passage was repeated in LB medium containing gradually increased concentrations of rifampicin until the latter reached 100 µg ml^{-1}. One of the colonies that grew on an LB agar plate containing 100 µg ml^{-1} rifampicin was randomly selected and named C312M. The genetic identity of C312M was verified by sequence analysis of the 16S rRNA gene as described previously (Zhang & Sun 2007) and by sequence analysis of PCR products obtained with the primer pairs Dps2F (5′-ATG TTG TCA CAA GCT ATG GTT-3′)/Dps2R (5′-CAC AGC TTG GGT ATC CAT TA-3′) and SodF (5′-ATG TCA TTC GAA TTA CCA GCT-3′)/SodR (5′-TTT TGC TAG GTT TTC TGC AAC-3′), which are specific to the ferritin and superoxide dismutase genes of *V. anguillarum*, respectively (Naka et al. 2011).

Fish

Japanese flounder *Paralichthys olivaceus* (average ± SE = 12.6 ± 0.6 g) were purchased from local fish farms and acclimatized in the laboratory for 2 wk before experimental manipulation. Fish were fed daily with commercial dry pellets and maintained at ~22°C in sand-filtered and activated carbon-absorbed seawater that was changed twice daily. Before the experiment, fish (5%) were randomly sampled for the examination of bacterial recovery from blood, liver, kidney, and spleen by the plate-count method described by Zhang et al. (2008), and 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 (5%) from the batch were negative for serum antibodies against *Vibrio anguillarum*. For tissue examinations, fish were sacrificed with an overdose of tricaine methanesulfonate (Sigma) as described previously (Wang et al. 2009).

Virulence of C312M

The median lethal dose (LD_{50}) was determined as described previously (Wang et al. 2009). To examine bacterial dissemination in tissues, 2 groups (N = 70) of Japanese flounder were infected via intraperitoneal (i.p.) injection with the same dose (10^6 colony-forming units, CFU) of C312 or C312M. For each

infection, liver, kidney, spleen, and blood were taken aseptically from fish (5 each time) at 1, 2, 3, 4, 6, 9, 12, 14, 16, 18, and 20 d post-infection (dpi). The tissues were homogenized in phosphate-buffered saline (PBS). The homogenates and blood were serially diluted and plated in triplicate in LB agar plates supplemented with ampicillin (selection marker for C312 and C312M). The plates were incubated at 28°C for 48 h, and the colonies that appeared were enumerated. The genetic nature of the colonies was examined as described above.

Virulence stability of C312M

To examine the stability of C312M under *in vitro* conditions, the bacterium was cultured in LB medium to an OD_{600} of 0.8 and diluted in fresh LB to 10^5 CFU ml^{-1}. The diluted culture was incubated at 28°C to OD_{600} 0.8; 1 portion of the cell culture was used for LD_{50} examination, while another portion of the cell culture was diluted in fresh LB medium to 10^5 CFU ml^{-1} and incubated at 28°C to OD_{600} 0.8 as described above. The subculture was repeated 10 times. To examine the stability of C312M under *in vivo* conditions, the bacterium was cultured in LB medium to an OD_{600} of 0.9, and the cells were washed with PBS and resuspended in PBS to 10^9 CFU ml^{-1}. Cell suspension (100 µl) was injected i.p. into flounder. At 2 dpi, liver, spleen, and kidney were taken under aseptic conditions and homogenized in sterile PBS. The homogenates were mixed at an equal volume, and 100 µl of the mixture was used to start the second round of infection by i.p. injection into naïve fish. This process was repeated 10 times. The homogenate mixtures of the fifth and tenth rounds of infection were plated on LB medium, and the recovered bacteria were examined for LD_{50} as described above.

Whole-cell protein production

C312 and C312M were cultured in LB medium to an OD_{600} of 1, and the cells were collected by centrifugation. Whole-cell proteins were prepared by lysing the cells in lysis buffer as described previously (Cheng et al. 2010). The concentration of the proteins was determined using the Bradford method with bovine serum albumin (BSA) as the standard. The proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the proteins were stained with Coomassie brilliant blue R-250 (Sangon).

Protease and siderophore production

Protease activity was determined with a modified LB medium prepared by mixing 4 g peptone, 1 g yeast extract, 0.1 g ferric phosphate, 15 g gelatin, and 20 g agar in 1 l of aged seawater. The pH of the medium was adjusted to 7.6, autoclaved at 112.6°C for 30 min, and then poured into Petri dishes. C312 and C312M were cultured in standard LB medium to an OD_{600} of 1. The cells were washed with PBS and resuspended in PBS to 10^8 CFU ml^{-1}. Ten microliters of the cell suspension was spotted on an agar plate prepared as above. The plate was incubated at 28°C for 36 h, and 25% trichloroacetic acid was added to the plate to induce color formation. Siderophore production was determined with the chrome azurol S (CAS) agar method (Silva-Stenico et al. 2005) by adding 50 µl of the above bacterial cell suspension into an Oxford cup placed on a CAS agar plate, followed by incubating the plate at 28°C for 5 d.

Preparation of vaccine-containing microspheres

For oral vaccination, C312M was encapsulated into alginate microspheres as follows. The bacterium was cultured in LB medium at 28°C to OD_{600} of 1. The cells were washed with and resuspended in PBS to 5×10^9 CFU ml^{-1}. Twenty milliliters of 3% (m/v) sodium alginate was mixed with 12 ml of bacterial suspension or PBS (control), and the mixture was emulsified by adding 800 ml of paraffin and 4 ml of Span-80. While stirring, 20 ml of 0.15 M $CaCl_2$ was added to the emulsion, and microspheres were collected by centrifugation at 1000 × *g* for 10 min. Marine fish feed (100 g; Shandong Sheng-suo Fish Feed Research Center) was mixed with vaccine-containing microspheres or the control microspheres. The prepared feed was then 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 3 d.

Vaccination

Flounder described above were randomly divided into 6 groups (N = 300) named A to F. For oral vaccination, Group A was fed for 3 d with feed containing C312M microspheres to the amount that the daily bacterial consumption was approximately 5×10^7 CFU $fish^{-1}$, while Group B (control) was similarly fed with feed containing control

microspheres. The amount of daily bacterial consumption was estimated as follows: as described in the previous subsection, 12 ml of bacterial suspension (5×10^9 CFU ml^{-1}) was mixed with 100 g feed; considering bacterial loss during the preparation process, the vaccine content in the feed pellet was estimated to be 5×10^8 CFU g^{-1}. The fish were fed about 0.2 g feed d^{-1}, which is equivalent to a daily bacterial consumption of ~5×10^7 CFU. For immersion vaccination, C312M was cultured in LB medium as described above and resuspended in seawater to 2×10^7 CFU ml^{-1}. Group C was immersed in C312M-containing seawater for 6 h, while Group D (control) was similarly treated in PBS-containing seawater. The fish were then moved to tanks containing fresh seawater and reared under normal conditions as described above. For oral plus immersion vaccination, Group E was vaccinated via oral feeding as described above and then immediately vaccinated via immersion as described above, while Group F (control) was orally vaccinated as described above for Group B and then bath vaccinated as described above for Group D. At 1 mo post-vaccination, 30 fish were taken from Groups A to F, respectively, and challenged via immersion with C312, and 30 fish were taken from Groups E and F and challenged similarly with *Vibrio anguillarum* 19264. After each challenge, the fish were monitored for mortality for 20 d, and dying fish were randomly selected for the examination of bacterial recovery from liver, kidney, and spleen as described previously (Zhang et al. 2008). Briefly, the tissues were homogenized in PBS, and the homogenates were plated in triplicate on LB agar plates. The plates were incubated at 28°C for 24 h. The genetic identity of the colonies that emerged on the plates was examined by PCR with the primer pairs Dps2F/Dps2R and SodF/SodR, as described above. Relative percent survival (RPS) was calculated according to the following formula: RPS = [1 − (% mortality in vaccinated fish/% mortality in control fish)] × 100 (Amend 1981). All vaccination trials were conducted in duplicate, and the mean values are shown in the results.

Infection of C312M into fish tissues following vaccination

Liver, spleen, kidney, and gut were taken from C312M- and PBS-vaccinated fish at 1, 2, 3, 4, 6, 8, 10, 12, 14, 16, 18, and 20 d after vaccination (5 fish per time point). Bacterial invasion into tissues was examined by determining bacterial recovery from each of the tissues as described above.

ELISA and serum bactericidal activity

Sera were collected from unvaccinated and vaccinated fish (5 at each time point) at 1 mo post-vaccination. Sera were diluted 64-fold in PBST (0.1% Tween-20 in PBS) containing 1% BSA. Serum antibodies against C312M were determined by ELISA as described previously (Sun et al. 2011). The assay was performed with sera from 5 fish as 5 individual samples. The serum bactericidal assay was performed as follows. C312 and C312M were cultured in LB medium to mid-logarithmic phase and resuspended in PBS to 10^6 CFU ml^{-1}. Ten microliters of bacterial suspension or PBS was mixed with or without 50 µl untreated serum or heat-treated (55°C for 30 min) serum in a total volume of 110 µl. The mixture was incubated at 30°C for 1 h and, after diluting in LB, plated on 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 as described above. The assay was performed with sera from 5 fish as 5 individual samples.

Statistical analysis

All statistical analyses were performed with analysis of variance (ANOVA) in the SPSS 17.0 package. In all cases, significance was defined as $p < 0.05$.

RESULTS

Characterization of C312M and C312

C312M was one of the mutants derived from C312 after 7 rounds of repeated selection of rifampicin resistance. To examine whether there was any difference between C312M and C312 in growth and protein production, the 2 strains were cultured in normal LB medium or in LB medium supplemented with the iron chelator 2,2′-dipyridyl (200 µM), which created an iron-deficient condition. The results showed that when cultured in standard LB medium, the growth of C312M was significantly ($p < 0.05$) slower than that of C312 at all the sample time points between 12 and 40 h, while when cultured in LB medium containing

2,2′-dipyridyl, the growth of C312M was significantly ($p < 0.05$) slower than that of C312 between 6 and 40 h (Fig. 1). Whole-cell protein production analysis showed that C312M exhibited a protein profile that, although bearing a general resemblance to that of C312, differed clearly from that of C312 in several bands (Fig. 2A). Compared to C312, C312M exhibited slightly stronger extracellular protease activity but less production of siderophores (Fig. 2B,C).

Virulence characteristics of C312M

When flounder were infected with C312M via immersion or oral feeding, 4×10^8 CFU of C312M caused no mortality. To compare the virulence of C312M and C312, flounder were infected with different doses of C312M or C312 via i.p. injection. Subsequent LD_{50} analysis showed that the LD_{50} of C312M and C312 were approximately 1.5×10^8 CFU fish^{-1} and 10^6 CFU fish^{-1}, respectively (Fig. A1 in the Appendix). To compare the tissue infectivity of C312M and C312, the strains were each inoculated via i.p. injection into flounder at the dose of 10^6 CFU, and bacterial recoveries from liver, spleen, kidney, and blood were examined at different time points. The results showed that live bacteria were recovered from both C312M- and C312-infected fish (Fig. 3). For C312M, live bacteria were recovered in all examined tissues at 1 dpi, and peak levels of recovery were reached at 2 to 3 dpi. After 3 dpi, the amounts of recovered C312M decreased with time until 14 dpi, at which time point no C312M was recoverable from any of the examined tissues (Fig. 3B). No mortality was observed in C312M-infected fish. In contrast, mortality began to occur at 5 dpi in C312-infected fish.

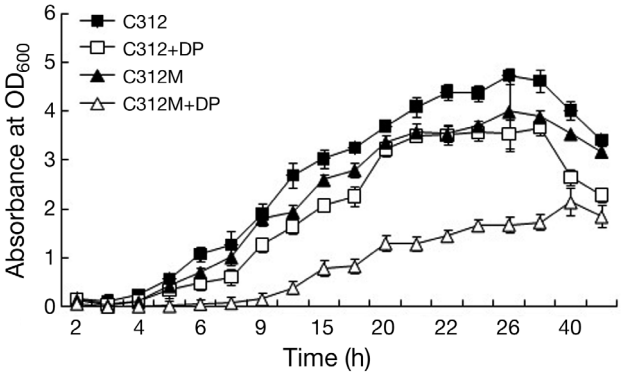

Fig. 1. *Vibrio anguillarum.* Growth curves of strains C312M and C312. C312M and C312 were cultured in Luria-Bertani medium in the absence or presence of 2,2′-dipyridyl (DP), and cell densities were determined at different time points by measuring absorbance at OD_{600}. Data are means ± SE (N = 3)

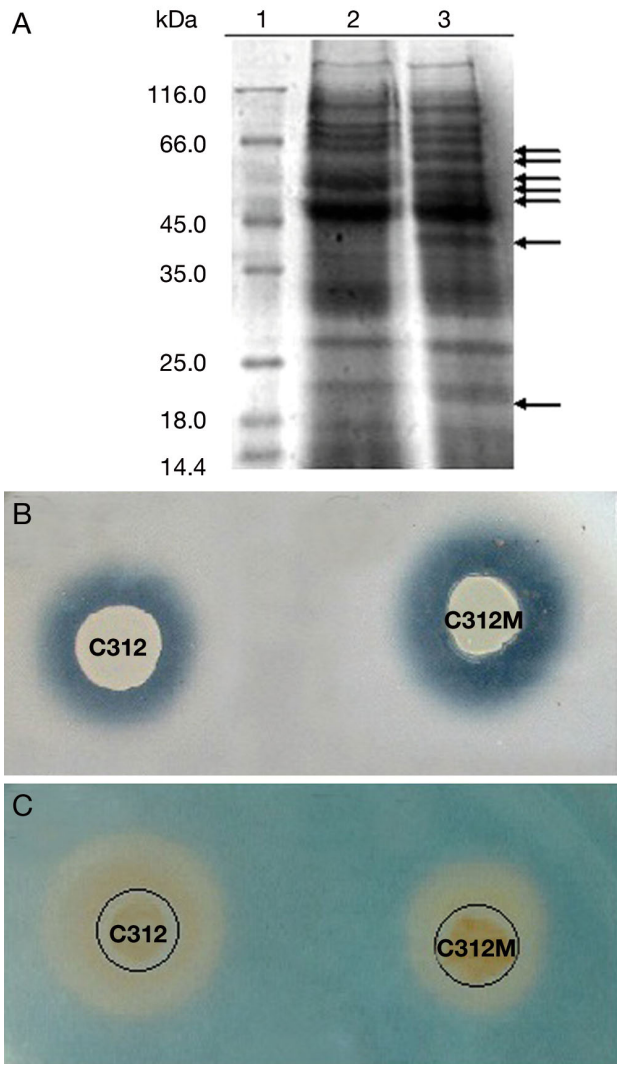

Fig. 2. *Vibrio anguillarum.* Analysis of (A) the protein expression profile, (B) extracellular protease activity, and (C) siderophore production of strains C312M and C312. In (A), whole-cell proteins from C312 (Lane 2) and C312M (Lane 3) were resolved by SDS-PAGE and viewed after staining with Coomassie brilliant blue. Arrows indicate representative bands that differ between C312M and C312. Lane 1: protein size markers. In (B), C312 and C312M were spotted on an LB agar plate containing gelatin, and 25% trichloroacetic acid was added to the plate after 36 h incubation at 28°C. In (C), C312 and C312M were added into Oxford cups (black circles) placed on a chrome azurol S agar plate, and the plate was incubated at 28°C for 5 d. For both (B) and (C), halos around the bacterial spots indicate positive results

In vivo and *in vitro* stability of C312M

To examine whether C312M could revert to wild type in the absence of selective pressures, the strain was successively cultured 10 times in LB medium without antibiotics, and the sub-cultures were exam-

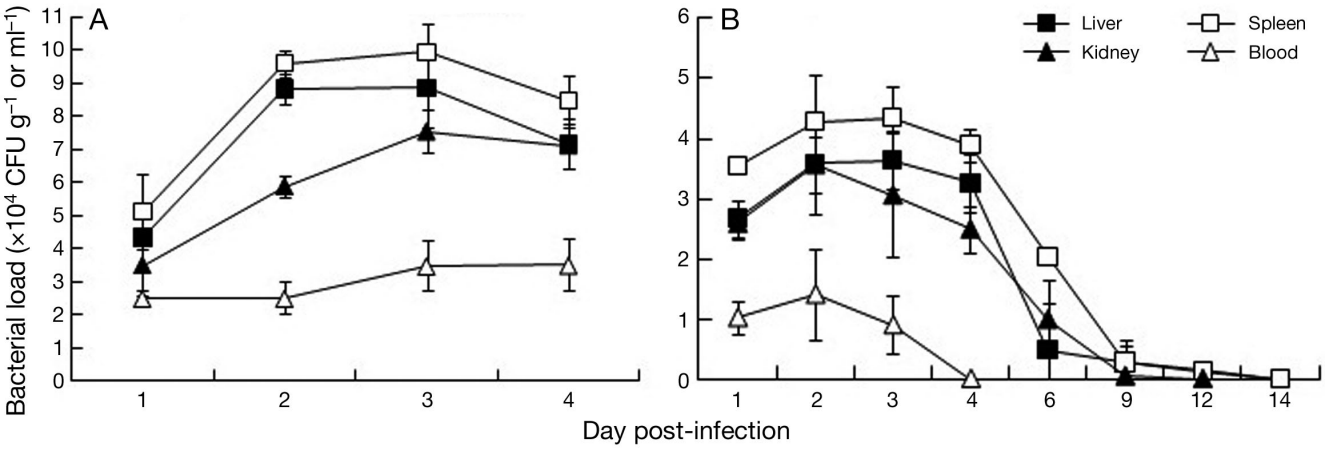

Fig. 3. *Paralichthys olivaceus* infected with *Vibrio anguillarum.* Tissue dissemination of strains (A) C312 and (B) C312M. Flounder were infected with 10^6 colony-forming units (CFU) of C312 or C312M via intraperitoneal injection. Bacterial recovery from the liver, spleen, kidney, and blood was determined at various time points. For C312, examination stopped on Day 4, since mortality began to occur on Day 5 post-infection. Data are means ± SE (N = 5)

ined for LD_{50}. The results showed that the LD_{50} of the fifth and tenth sub-cultures (1.5×10^8 CFU fish^{-1}) were the same as the LD_{50} of C312M prior to the passage. To examine the stability of C312M under *in vivo* conditions, C312M was passaged 10 times in flounder, and the C312M recovered from the tissues of infected fish was examined for LD_{50}. The results showed that the LD_{50} values of the C312M recovered at the fifth and tenth rounds of infection (1.4×10^8 CFU fish^{-1}) were comparable to that of C312M before infection.

Vaccination of flounder with C312M via oral, immersion, and oral plus immersion routes

Immunoprotective effect. Since C312M is highly and stably attenuated in virulence, we examined its potential as an oral, immersion, and oral plus immersion vaccine. For this purpose, flounder were vaccinated via oral, immersion, and oral plus immersion routes with C312M or PBS (control). The fish were challenged with C312 at 1 mo post-vaccination and monitored for mortality. The results showed that the cumulative mortalities (mean ± SE) were 27 ± 3, 20 ± 2, and 10 ± 0% in fish vaccinated with C312M via oral, immersion, and oral plus immersion routes, respectively, while the cumulative mortalities were 67 ± 3, 60 ± 5, and 63 ± 2% in fish vaccinated with PBS via oral, immersion, and oral plus immersion routes, respectively (Table 1). Hence, the protection rates, in terms of RPS, of C312M were 60, 67, and 84% as oral, immersion, and oral plus immersion vaccines, respectively. To examine whether C312M could induce protection against a heterologous strain, fish vaccinated with C312M and PBS via oral plus immersion routes were challenged with *Vibrio anguillarum* 19264. The results showed that *V. anguillarum* 19264 challenge caused cumulative mortality rates of 17 ± 2 and 63 ± 7% in C312M- and PBS-vaccinated fish, respectively, which corresponds to an RPS rate of 73%. Microbiological analysis showed that C312 and *V. anguillarum* 19264 were the only types of bacteria recovered from the liver, spleen, and kidney of dying fish challenged with C312 and *V. anguillarum* 19264, respectively.

Invasion and colonization of C312M in fish tissues after vaccination. The ability of C312M to invade into and colonize fish tissues following oral plus

Table 1. *Paralichthys olivaceus* infected with *Vibrio anguillarum.* Results of the vaccination experiment with C312M, a rifampicin-resistant *V. anguillarum* strain. Two strains of *V. anguillarum* (C312 and 19264) were used as the challenging organisms. Cum. mort.: cummulative mortality (mean ± SE) PBS: phosphate-buffered saline, used as a control; RPS: relative percent survival

Vaccine	Administration route	*Vibrio* strain	Cum. mort. (%)	RPS (%)
C312M	Oral	C312	27 ± 3	60
PBS	Oral	C312	67 ± 3	
C312M	Immersion	C312	20 ± 2	67
PBS	Immersion	C312	60 ± 5	
C312M	Oral plus immersion	C312	10 ± 0	84
PBS	Oral plus immersion	C312	63 ± 2	
C312M	Oral plus immersion	19264	17 ± 2	73
PBS	Oral plus immersion	19264	63 ± 7	

immersion vaccination was determined by bacterial recovery analysis, which showed that C312M was recovered from the gut, liver, kidney, and spleen of the fish vaccinated with C312M (Fig. 4) but not from the same tissues of the control fish vaccinated with PBS (data not shown). The amounts of recovered C312M were highest in the first 3 d after vaccination and then declined with time until 10 to 14 d post-vaccination. After 14 d post-vaccination, no C312M was detected in the examined tissues.

C312M-induced serum antibodies: production and bactericidal activity. ELISA was performed to examine serum antibody production induced by C312M. The results showed that at 1 mo post-vaccination, serum antibodies against C312M were detected in fish vaccinated with C312M via oral, immersion, and oral plus immersion routes (Fig. 5). To examine the bactericidal effect of serum from vaccinated and unvaccinated fish, sera from fish vaccinated with C312M and PBS (control) were incubated with C312 or *Vibrio anguillarum* 19264. Subsequent analysis of bacterial survival indicated that the survival rates of C312 and *V. anguillarum* 19264 in the sera of C312M-vacinated fish were significantly ($p < 0.01$) reduced to levels of 33.3 and 41.9%, respectively, of those in the sera of the control fish (Fig. 6). When the sera were heated before incubation with C312 and *V. anguillarum* 19264, the survival rates of C312 and *V. anguillarum* 19264 in the sera of C312M-vacinated fish were comparable to those in the sera of the control fish.

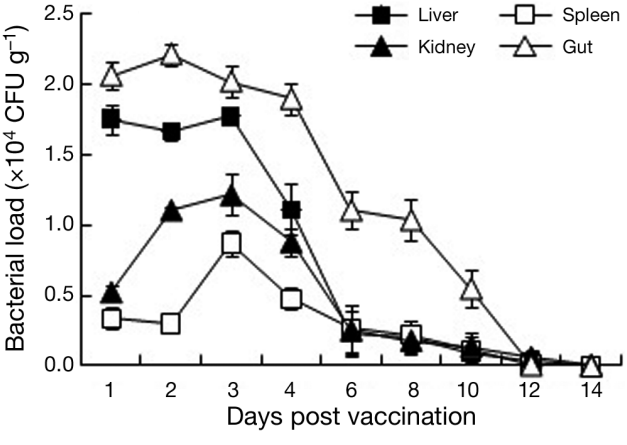

Fig. 4. *Paralichthys olivaceus* infected with *Vibrio anguillarum*. Invasion of strain C312M into fish tissues following vaccination via immersion and oral feeding. Gut, liver, kidney, and spleen were taken at different time points after vaccination and determined for bacterial recovery. Data are means ± SE (N = 5)

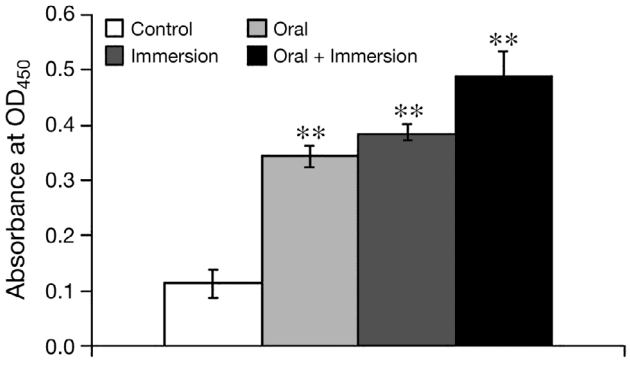

Fig. 5. *Paralichthys olivaceus* infected with *Vibrio anguillarum*. Strain C312M-induced serum antibody production. Sera were collected from unvaccinated fish (control) and fish vaccinated with C312M via oral, immersion, and oral plus immersion routes, and anti-C312M antibodies were determined by ELISA. Data are means ± SE (N = 5). **$p < 0.01$

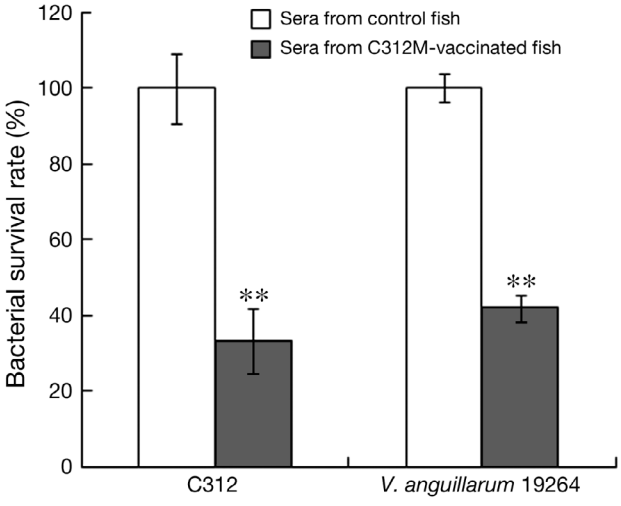

Fig. 6. *Paralichthys olivaceus* infected with *Vibrio anguillarum*. Serum bactericidal activity. Sera were collected from flounder vaccinated with or without (control) strain C312M, and serum bactericidal activity against C312 and *V. anguillarum* 19264 was determined. The bactericidal activity of control serum was set as 100%. Data are means ± SE (N = 5). **$p < 0.01$

DISCUSSION

In the present study, we selected and characterized a rifampicin-resistant mutant *Vibrio anguillarum*, C312M. We found that compared to the wild type, C312M was moderately retarded in growth when cultured in the rich LB medium and drastically retarded when cultured in the presence of the iron chelator 2,2′-dipyridyl, suggesting that C312M was impaired in growth, particularly under conditions of

iron depletion. For pathogenic bacteria, mutation to an antibiotic-resistant phenotype is sometimes associated with alteration in protein expression (Zhang et al. 2006, LaFrentz et al. 2008, Sun et al. 2010, Hu et al. 2012). In the present study, we found that the whole-cell protein profile of C312M differed from that of C312 in several places. These results suggest that acquisition of rifampicin resistance in C312M causes changes in the expression of some proteins that may be responsible for the reduced growth rate of C312M, especially under conditions of nutrient deficiency.

Virulence analysis showed that C312M exhibited an LD_{50} that was more than 100 times higher than that of the wild type, suggesting that C312M is highly attenuated in general virulence. It is possible that virulence attenuation was at least in part due to the altered protein expression and disregulated production of proteases and siderophores as observed with C312M. Consistent with the results of LD_{50} analysis, tissue infectivity analysis showed that when inoculated via i.p. injection into flounder, both C312M and C312 were recovered from the liver, spleen, kidney, and blood of the infected fish; however, unlike C312, which induced mortality at 5 dpi, C312M induced no mortality, and no C312M could be recovered from the tissues after 14 dpi. These results indicate that C312M, though highly reduced in virulence compared to C312, retained residual infectivity and was able to disseminate into and colonize host tissues for a short period of time. The observation that when sub-cultured 10 times *in vitro* in LB medium without rifampicin or being passed 10 rounds *in vivo* in live flounder, C312M exhibited no significant changes in LD_{50} suggests that C312M was stable under the experimental conditions and did not revert to the virulent wild-type form. These results provided a safety basis for subsequent tests of C312M as a live vaccine.

Compared to injection vaccination, which is costly and limited to animals of certain sizes, immersion and oral vaccinations are cheaper and inflict no stress to the target animals and hence are considered ideal vaccination approaches (Sommerset et al. 2005, Plant & Lapatra 2011). In the present study, since C312M was stably reduced in virulence, we examined its potential as a live vaccine administered via immersion and oral routes. We found that following challenge with C312, fish vaccinated with C312M via oral, immersion, and oral plus immersion routes exhibited RPS rates of 60, 67, and 84%, respectively. These results indicate that C312M elicited effective protection in the form of oral, immersion, and, in particular, oral plus immersion vaccines. The observation that following challenge with *Vibrio anguillarum* 19264, C312-vaccinated fish exhibited an RPS rate of 73% suggests that C312M induced protective immunity against both homologous and heterologous *V. anguillarum* strains. Since C312M was recovered from the liver, kidney, and spleen in the 2 wk post-vaccination, it is likely that following vaccination, the residual virulence of C312M enabled the bacterial cells to invade into the tissues of vaccinated fish, thereby inducing protective immune response.

At 1 mo post-vaccination, serum antibodies against C312M were detected in C312M-vaccinated fish, suggesting that C312M induced B cell-mediated humoral immunity. Bactericidal activity analysis showed that serum from C312M-vaccinated fish exhibited significantly stronger killing effects against both C312 and *Vibrio anguillarum* 19264. These results were in line with the cross-protection observed with C312M against C312 and *V. anguillarum* 19264 and suggest that antibodies probably play an important role in the immunoprotection induced by C312M. Since heat treatment, which inactivated complement proteins, reduced the bactericidal activity of the serum from C312M-vaccinated fish to levels comparable to those of control serum, the enhanced bactericidal activity of C312M-induced serum was most likely complement-mediated.

In summary, compared to the wild-type strain, the rifampicin-resistant *Vibrio anguillarum* strain, C312M is defective in growth, altered in protein production profile, and highly attenuated in virulence. However, C312M possesses residual virulence that enables the bacterium to disseminate into host tissues following oral and immersion vaccination. C312M can confer effective protection in flounder against C312 and a heterologous *V. anguillarum* strain when administered via oral and immersion routes. These results indicate that C312M is a good vaccine candidate that may be used in aquaculture against *V. anguillarum*-related vibriosis.

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Appendix 1.

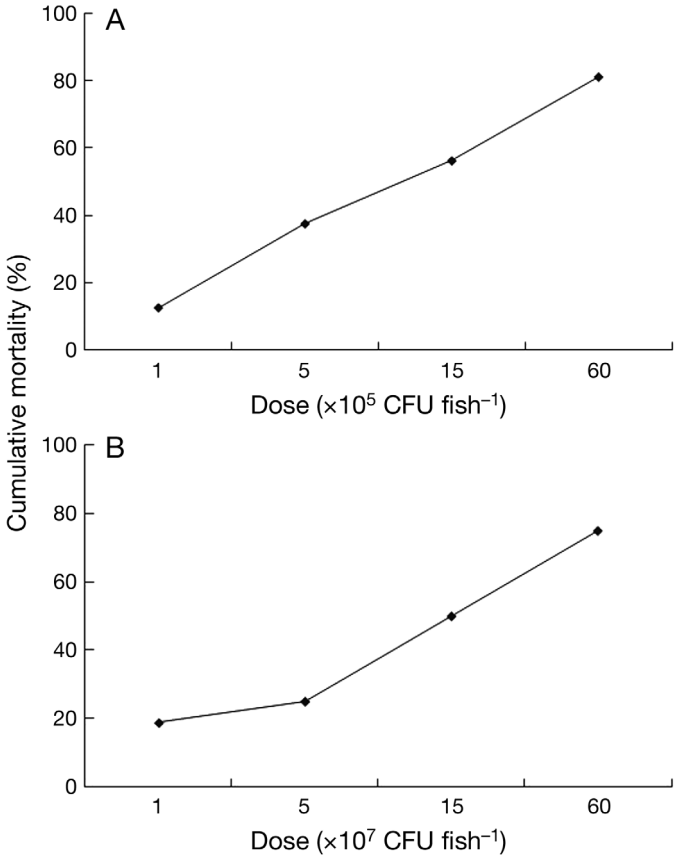

Fig. A1. *Paralichthys olivaceus* infected with *Vibrio anguillarum*. Mortality curves of flounder infected with various doses of *V. anguillarum* strains (A) C312 and (B) C312M. Flounder were infected via intraperitoneal injection, and the accumulated mortalities were monitored for a period of 20 d

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