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A cohabitation challenge to compare the efficacies of vaccines for bacterial kidney disease (BKD) in chinook salmon *Oncorhynchus tshawytscha*

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ABSTRACT: The relative efficacies of 1 commercial and 5 experimental vaccines for bacterial kidney disease (BKD) were compared through a cohabitation waterborne challenge. Groups of juvenile chinook salmon *Oncorhynchus tshawytscha* were vaccinated with one of the following: (1) killed *Renibacterium salmoninarum* ATCC 33209 (Rs 33209) cells; (2) killed Rs 33209 cells which had been heated to 37°C for 48 h, a process that destroys the p57 protein; (3) killed *R. salmoninarum* MT239 (Rs MT239) cells; (4) heated Rs MT239 cells; (5) a recombinant version of the p57 protein (r-p57) emulsified in Freund's incomplete adjuvant (FIA); (6) the commercial BKD vaccine Renogen; (7) phosphate-buffered saline (PBS) emulsified with an equal volume of FIA; or (8) PBS alone. Following injection, each fish was marked with a subcutaneous fluorescent latex tag denoting its treatment group and the vaccinated fish were combined into sham and disease challenge tanks. Two weeks after these fish were vaccinated, separate groups of fish were injected with either PBS or live *R. salmoninarum* GL64 and were placed inside coated-wire mesh cylinders (liveboxes) in the sham and disease challenge tanks, respectively. Mortalities in both tanks were recorded for 285 d. Any mortalities among the livebox fish were replaced with an appropriate cohort (infected with *R. salmoninarum* or healthy) fish. None of the bacterins evaluated in this study induced protective immunity against the *R. salmoninarum* shed from the infected livebox fish. The percentage survival within the test groups in the *R. salmoninarum* challenge tank ranged from 59% (heated Rs MT239 bacterin) to 81% (PBS emulsified with FIA). There were no differences in the percentage survival among the PBS-, PBS/FIA-, r-p57- and Renogen-injected groups. There also were no differences in survival among the bacterin groups, regardless of whether the bacterial cells had been heated or left untreated prior to injection.

KEY WORDS: *Renibacterium salmoninarum* · Bacterial kidney disease · Vaccine efficacy · Cohabitation challenge

INTRODUCTION

Renibacterium salmoninarum, the etiologic agent of bacterial kidney disease (BKD), is one of the most important bacterial pathogens of salmonids, accounting for as much as 80% of the mortality in cultured Pacific salmonids, *Oncorhynchus* spp. (Evenden et al. 1993). Efforts to control the spread of this organism have included brood stock segregation (Pascho et al. 1991) to interrupt the vertical transmission of *R. salmoninarum*, and the use of the chemotherapeutants, particularly erythromycin, to reduce or eliminate infection (Elliott et al. 1989). However, outbreaks of BKD persist despite these practices. When avoidance or treatment are impossible, vaccination may represent the most effective control method for some fish pathogens. Vaccination of fish has been done for about 60 yr and today there are commercially available-

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vaccines for several bacterial diseases of fish, including the salmonid pathogens *Yersinia ruckeri*, *Aeromonas salmonicida*, *Listonella* (*Vibrio*) *anguillarum* and *Vibrio salmonicida* (Schnick et al. 1997). However, efficacious vaccines for many other fish pathogens remain in the developmental stages.

Conventional approaches to fish vaccination do not effectively confer protection against *Renibacterium salmoninarum*. Salmonids respond to bacterins made from killed *R. salmoninarum* (Evelyn 1971, Paterson et al. 1985), but there is no clear correlation between a fish's ability to produce antibodies against *R. salmoninarum* bacterin and protection from BKD. In fact, groups of fish vaccinated with whole *R. salmoninarum* cells or isolated proteins may actually have a greater susceptibility to BKD than groups that were not vaccinated (Pascho et al. 1997). The lack of protection may be due in part to the 57 kDa protein (p57) of *R. salmoninarum*. p57 is highly expressed in virulent *R. salmoninarum* strains as both secreted and cell-surface forms. Concentrations of secreted p57 in the serum of infected fish can be as much as 1 mg ml^{-1} (Turaga et al. 1987) and p57 is highly immunogenic in salmonids (Alcorn & Pascho 2002). A high concentration of p57 in the serum of an infected fish, coupled with an elevated antibody response to the protein, may result in immune complexes, which are believed to contribute to the glomerulonephritic lesions characteristic of BKD (Kaattari & Piganelli 1997).

Although the exact functions of the p57 protein have not been determined, it is thought to be a virulence factor of *Renibacterium salmoninarum*. The MT239 strain, which produces the p57 protein but lacks the cell-bound form, is less virulent than wildtype strains (Bruno 1988, Senson & Stevenson 1999). Several of the immunosuppressive properties of *R. salmoninarum* are attributed to the p57 protein, including leucoagglutination (Wiens & Kaattari 1991, Fredriksen et al. 1997) and reduction of the antibody response (Turaga et al. 1987, Fredriksen et al. 1997). Exposure of salmonid macrophages to p57 can reduce their bactericidal (Siegel & Congleton 1997) and respiratory burst activity (Densmore et al. 1998). Molecules of p57 on the bacterial cell surface may also block the fish's immune response to other cell-surface antigens. Wood & Kaattari (1996) reported that removal of p57 by heating the cells to 37°C for 10 h enhanced the immunogenicity of an *R. salmoninarum* bacterin. They reported a 20-fold increase in the antibody response of chinook salmon injected with the modified cells compared to the response of fish injected with a bacterin made from the wildtype bacterium. The authors speculated that the increased antibody response may reflect the loss of a factor (presumably the p57 protein) that either blocks antibody synthesis, or obstructs immune reactions to other, carbohydrate antigens. The use of other, non-p57, cell surface antigens provides an intriguing vaccination strategy. The hypothesized role of p57 in immune complex formation and bacterial surface-antigen blocking led us to examine the strategy of immunizing fish with *R. salmoninarum* cells lacking the cell-associated p57.

The long-term goal of this project is to provide fish culturists with a vaccine against *Renibacterium salmoninarum*. The proposed project compared the relative efficacies of 5 experimental vaccines and 1 commercial vaccine against *R. salmoninarum* to protect juvenile chinook salmon during a waterborne challenge with a wildtype *R. salmoninarum*.

MATERIALS AND METHODS

Fish. Fish for this study were reared from eggs obtained from brood year 1998 and 2000 Root River (Wisconsin, USA) fall chinook salmon *Oncorhynchus tshawytscha*. Chinook salmon, brood year 2000, from the Abernathy Salmon Culture Technology Center (Washington, USA) were also used in this study. The presence and levels of *Renibacterium salmoninarum* in the spawning parental fish were determined by testing samples of kidney tissue and ovarian fluid using either the enzyme-linked immunosorbent assay (ELISA, Pascho et al. 1991) or the membrane filtration fluorescent antibody test (MF-FAT, Elliott & McKibben 1997). Eyed eggs from 5 mating pairs, in which both parents were categorized as negative for *R. salmoninarum* infection, were transferred to the Western Fisheries Research Center (US Geological Survey), Seattle, Washington, USA. The fish were reared in sand-filtered, UV-treated Lake Washington water at 12°C throughout the study. For each salmon stock and brood year, the progeny fish were combined at the fry stage and reared in 712 l circular tanks. The fish were fed ad libitum daily with a pelleted, semi-moist commercial diet (BioOregon).

***Renibacterium salmoninarum* whole cell bacterins.** Two strains of *Renibacterium salmoninarum* were used in this study: (1) ATCC strain 33209 (Rs 33209), which has cell-surface bound p57 (Wiens & Kaattari 1989) and (2) the MT-239 strain (Rs MT239), which produces the p57 protein but does not attach it to the cell surface (Bruno 1988). Each strain was grown in KDM 2 broth at 15°C for about 14 d. The bacteria were washed 3 times and resuspended in phosphate-buffered saline (PBS). One half of the volume of both strain preparations was heated to 37°C for 48 h and washed twice with PBS to remove residual p57 (Wood & Kaattari 1996). The 4 preparations were then inactivated in 0.85% (w/v) NaCl containing 0.4% (v/v) formalin for 24 h at 4°C.

The concentration of the 4 preparations were equilibrated from the original broth cultures on the basis of total cell counts by the MF-FAT and standard quantitative microbiological methods using KDM 2 agar plates.

The successful removal of p57 from the heated Rs 33209 and Rs MT239 cellular preparations was confirmed by Western blot analysis. All labware and reagents were purchased from BioRad Laboratories unless otherwise stated. The Mini-PROTEAN II was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the manufacturer's recommendations. The 4 inactivated bacterial preparations were diluted 1:50 with PBS. PBS solutions containing 13.5 µg ml^{-1} of recombinant p57 (see below) and each of the 4 bacterial preparations were separately mixed with an equal amount of SDS-PAGE sample buffer, and incubated at 95°C for 4 min. Then, 20 µl of each sample was separated by SDS-PAGE for 45 min at 200 V, 120 mA, in a discontinuous 0.125 M Tris, pH 6.8, 5% acrylamide stacking gel and 0.375 M Tris, pH 8.8, 12% separating gel. The separated proteins were transferred to a 0.2 µm nitrocellulose membrane using the Mini Trans-blot cell according to the manufacturer's recommendations. The free binding sites on the membrane were blocked for 1 h with milk protein solution (Kirkegaard and Perry Laboratories). The membrane was incubated overnight at 4°C in a monoclonal antibody solution: 1 part culture supernatant from each of 2 mouse hybridomas that produce antibody to different epitopes of p57 (3H1 and 4D3, Wiens & Kaattari 1991), and 2 parts 15 mM Tris buffered saline containing 0.05% v/v Tween 20 (TBS + Tween 20). The membrane was washed (3 × 20 min) with TBS + Tween 20. The membrane was incubated for 1 h at room temperature in TBS + Tween 20 containing (1:1000) horseradish peroxidase conjugated goat-anti-mouse IgG (H + L). The membrane was washed as described above and exposed to the substrate-chromogen (4-chloro-1-naphthol) solution for 5 min. Color development was halted by rinsing the membrane with water.

Commercial bacterin. The commercial bacterin, Renogen, was purchased from Aqua Health. Renogen is a live cell vaccine containing a bacterium that shares antigenic determinants with *Renibacterium salmoninarum* according to the manufacturer. It was prepared and administered according to the manufacturer's instructions.

Recombinant p57. The entire DNA sequence for the p57 protein of *Renibacterium salmoninarum* (Chien et al. 1992) was inserted into a high-expression plasmid (pET30, Novagen). For large-scale production of the recombinant p57 protein (r-p57), the pET30-p57 plasmid was transferred to *Escherichia coli* HMS174 (DE3) for growth in 100 l of Terrific broth containing 25 µg ml^{-1} kanamycin. Expression of the r-p57 was induced by the addition of 1 mM isopropyl β-D-thiogalactopyranoside (IPTG). 4 h post-induction, the culture was harvested and the 420 g wet weight cell pellet was stored overnight at −70°C. To harvest the insoluble inclusion bodies, the bacterial cells were lysed by a combination of lysozyme treatment and sonication. After thawing, the cell pellet was suspended in 20 mM Tris buffer, pH 8.0, containing 500 mM NaCl, 5 mM imidazole, 1 mM EDTA and 2 mg ml^{-1} lysozyme. The suspension was incubated for 1 h at room temperature; then, Triton X-100 was added to a final concentration of 0.5% (v/v). The suspension was incubated for an additional 30 min and then sonicated to ensure cell lysis. The inclusion bodies and other cellular debris were concentrated by centrifugation at 10 000 × *g*, after which the 'resuspend-sonicate-spin' cycle was repeated 6 times to generate washed inclusion bodies. The insoluble material was suspended in 730 ml of 20 mM Tris, pH 8.0, containing 500 mM NaCl and 5 mM imidazole, and stored at −80°C.

To prepare the r-p57 stock solution used in this study, an aliquot of the purified inclusion bodies was solubilized in 200 ml of Voller PBS containing 6 M urea at a concentration of 1.99 mg ml^{-1}. The protein solution was sequentially dialyzed against Voller PBS containing decreasing concentrations of urea; 4 M, 2 M, 1 M and no urea. At each urea concentration, the r-p57 was dialyzed at 4°C with stirring for 24 h. The r-p57 solution was 0.2 µm filtered to sterilize, and aliquots were stored at −80°C. Protein concentration was determined with the Bradford protein assay (BioRad) using bovine serum albumin as the standard. Serological reactivity to the monoclonal antibodies 3H1 and 4D3, which are specific to separate epitopes of the native p57 protein (Wiens & Kaattari 1991), was determined by Western blot.

Selection of a bacterin dose. The relative immune responses of groups of brood year 1998 Root River chinook salmon to different concentrations of the wildtype bacterin (Rs 33209) were used to select a single test dose for all of the bacterins in the challenge study. Six groups of 160 chinook salmon, mean weight of 19 g, were injected intraperitoneally (IP) with either 100 µl of 50 µg r-p57 emulsified with Freund's incomplete adjuvant (FIA, positive control), or 500 µl of 1 of 5 concentrations of the Rs 33209 bacterin in PBS. The amounts of the bacterin injected into fish were 5×10^9, 5×10^8, 5×10^6, 5×10^5 or 5×10^3 cells. Each group of fish was split into 2 subgroups of 80 fish each and placed in replicate 369 l tanks.

Fish from each subgroup were bled prior to immunization (prebleed), and biweekly between 3 and 13 wk after immunization. Ten fish were captured by repeated dip netting from each replicate tank of each

injection group and overdosed with MS222. Blood samples were collected in a heparinized Natelson capillary tube after the caudal peduncle was severed. The blood was transferred to a capped tube and incubated overnight at 4°C. After centrifugation of the blood samples at 10 000 × *g* for 10 min at 4°C, the plasma samples were collected and stored at –80°C.

The antigen-specific antibody response of the fish in each subgroup was determined by a quantitative ELISA based on coating the microplate well surfaces with r-p57 as a capture antigen (Alcorn & Pascho 2000). Briefly, a standard curve was constructed by relating multiple dilutions of a high titer rainbow trout antiserum to r-p57 to their resulting ELISA absorbance values. The ELISA absorbance values produced by single dilutions of the test plasma samples were then compared to the standard curve to determine the anti-p57 antibody concentration. Plasma samples that had anti-p57 antibody activity greater than the cutoff value were considered to be from responder fish and were used for data analysis. The cutoff value was considered to be the mean anti-p57 antibody activity of the 'prebleed' fish plus 2 standard deviations of the mean. The dose of Rs 33209 that elicited the greatest antibody response among responder fish was selected as the experimental dose for all bacterins.

Immunization of chinook salmon. Five groups of 200 Root River chinook salmon, brood year 2000 fish weighing approximately 10 g, each received a 500 µl IP injection of either PBS or one of the *Renibacterium salmoninarum* bacterins. The concentration of bacterial cells in each of the *R. salmoninarum* bacterins was equal and the dose was determined as described above. Two hundred chinook salmon were injected IP with 100 µl of 50 µg r-p57 emulsified with FIA. Two hundred chinook salmon were vaccinated with Renogen according to the manufacturer's recommendation (Aqua Health). Following vaccination, the fish received a subcutaneous latex tag (New West Technologies) in the anal, dorsal or caudal fin to designate the treatment group. After injection and marking, 100 fish from each vaccination group were put in each of two 1870 l circular tanks. The fish were maintained at 12°C throughout the remainder of the experiment.

Cohabitation challenge. In the cohabitation challenge, vaccinated test fish were exposed to *Renibacterium salmoninarum* by introduction of fish that had been previously injected with the live bacterium. During the normal course of an infection, injected fish shed *R. salmoninarum* into the water, which would potentially infect the test fish. The third passage of an *R. salmoninarum* strain originally isolated from an adult chinook salmon in Lake Michigan (Rs GL64) was used as the challenge bacterium. The challenge culture was grown in KDM 2 broth at 15°C for 10 d, then washed 3 times by centrifugation in PBS and resuspended in PBS. The bacterial cell concentration was determined by total cell counts using the MF-FAT, and colony-forming units by bacteriological culture on KDM 2 agar plates.

For the cohabitation challenge, exposure of the vaccinated fish to waterborne *Renibacterium salmoninarum* was to begin as they were undergoing their maximal immune response. While determining the optimal bacterin dose, the maximum antibody response in chinook salmon occurred 9 wk after injection with 5×10^9 formalin-inactivated *R. salmoninarum*. In a separate study, peak modulation of several immune functions occurred 2 to 4 wk after injection of the same concentration of *R. salmoninarum* Rs 33209 bacterin (data not shown). Based on these results, the potential for a *R. salmoninarum* bacterin to provide protection during a *R. salmoninarum* challenge was expected to begin about 4 to 9 wk after vaccination, depending on what immune functions were important for protection. In a previous study, fish injected with 1.3×10^6 *R. salmoninarum* began to shed detectable concentrations of bacteria after about 3 wk (McKibben & Pascho 1999). Therefore, livebox Abernathy strain chinook salmon were injected with *R. salmoninarum* 2 wk after completion of vaccination and marking of the test fish. The exposure of the test fish to the challenge bacteria was expected to begin about 5 wk after the test fish had been vaccinated.

Groups of 100 Abernathy chinook salmon, approximate size 10 to 15 g, were adipose fin-clipped and injected IP with either PBS or PBS containing 1×10^6 Rs GL64. Eighty fish (10% of the test population size) from the *Renibacterium salmoninarum*-injected group were equally distributed among 5 coated-wire mesh cylinders (liveboxes) in 1 of the challenge tanks containing the test fish (*R. salmoninarum* challenge tank). An equal number of PBS-injected fish was placed in 5 liveboxes in the other tank of test fish (sham challenge tank). The cylindrical liveboxes were approximately 25 cm in diameter and as deep as the water column, about 40 cm. The remaining injected fish were placed in separate tanks as reserves. Mortalities in both tanks were recorded for 285 d. Periodically, the anterior kidneys of dead fish were cultured to test for *R. salmoninarum* infection. Any mortalities among the livebox fish were replaced by cohort fish from the respective reserve tank.

Survival statistics were calculated by Kaplan-Meier analysis. Differences between the percentage survival of fish in each of the treatment groups were determined by the logrank comparison. Statistical significance was set at $p \leq 0.05$.

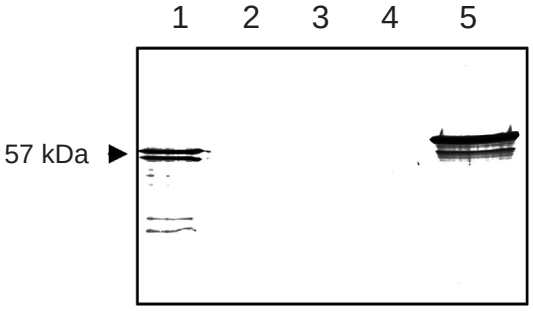

Fig. 1. Western blot analysis of the *Renibacterium salmoninarum* cellular preparations of strains ATCC 33209 (Rs 33209) and MT239 (Rs MT239) before, and after, heating at 37°C for 48 h. The recombinant p57 protein was included. Separated proteins were reacted with 2 mouse monoclonal antibodies (3H1 and 4D3) that recognize different epitopes of the p57 protein. Lanes: (1) Rs 33209, (2) heated Rs 33209, (3) Rs MT239, (4) heated Rs MT239, (5) recombinant p57 protein

RESULTS

Production of bacterins

Heating *Renibacterium salmoninarum* cells to 37°C for 48 h removed p57 from the Rs 33209 preparation (Fig. 1). The absence of p57 bands in the lane loaded with unheated *R. salmoninarum* MT239 was expected. Whereas this strain produces the p57 protein, it lacks p57 on the cell surface (Bruno 1990, Daly & Stevenson 1990). Thus, only very small amounts of the protein within the bacterial cells would remain for the analysis after the wash steps.

The recombinant p57 purified from lysed *Escherichia coli* cells was recognized by at least 1 of the monoclonal antibodies 3H1 and 4D3 (Fig. 1). The principle band in the r-p57 sample is slightly heavier than 57 kDa because of a histidine-tag on the recombinant protein.

Selection of a bacterin dose

Among the groups of chinook salmon injected with the Rs 33209 bacterin, only the fish injected with the 5×10^9 *Renibacterium salmoninarum* cells produced an antibody response similar to that of the fish injected with the r-p57 (Fig. 2). The peak antibody response of fish injected with 5×10^9 *R. salmoninarum* cells or r-p57 occurred about 9 wk after injection and the magnitudes of the responses were similar. A detectable antibody response was also observed among the fish injected with 5×10^8 *R. salmoninarum* cells. However, the magnitude of the antibody response to this lower concentration was lower and the peak response occurred about 7 wk after injection. Injection of most fish with less than 5×10^8 bacterin cells failed to elicit measurable antibody.

Cohabitation challenge

On the basis of testing water samples by the MF-FAT, high concentrations of *Renibacterium salmoninarum* were present in the water of the challenge tank 38 d after introduction of the infected fish (Fig. 3). The experiment was terminated 285 d after the introduction of the livebox fish.

At the termination of the experiment, there had been a total of 258 mortalities in the liveboxes: 15 among the sham injected fish and 243 among the *Renibacterium salmoninarum*-injected fish. Kidney tissue samples from all of the sham-injected livebox fish and some of the *R. salmoninarum*-injected livebox fish were

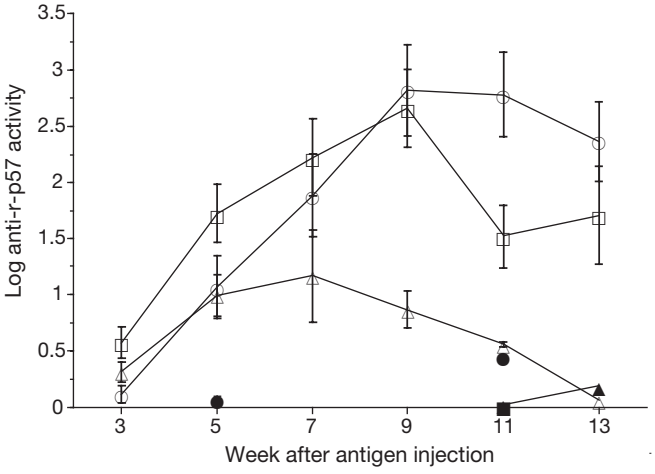

Fig. 2. Mean (±SE, n = 10) antibody response of chinook salmon injected with r-p57 (○), or 5×10^9 (□), 5×10^8 (△), 5×10^6 (●), 5×10^5 (■), or 5×10^3 (▲) untreated Rs 33209 cells

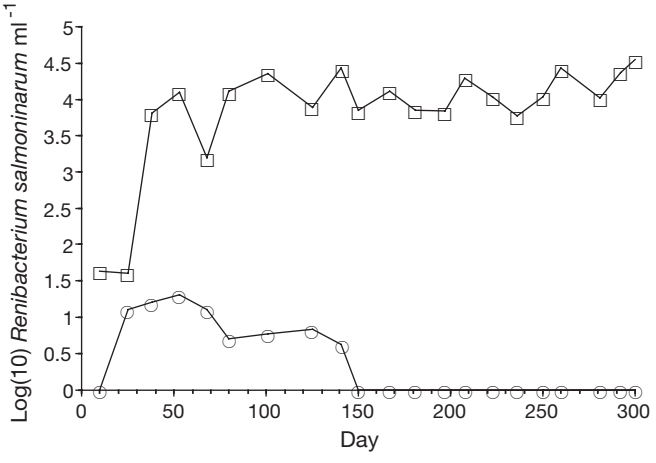

Fig. 3. Concentration of *Renibacterium salmoninarum* in the rearing water of the sham (○) and *R. salmoninarum* (□) challenge tanks after the introduction of livebox chinook salmon injected with either PBS or *R. salmoninarum*, respectively

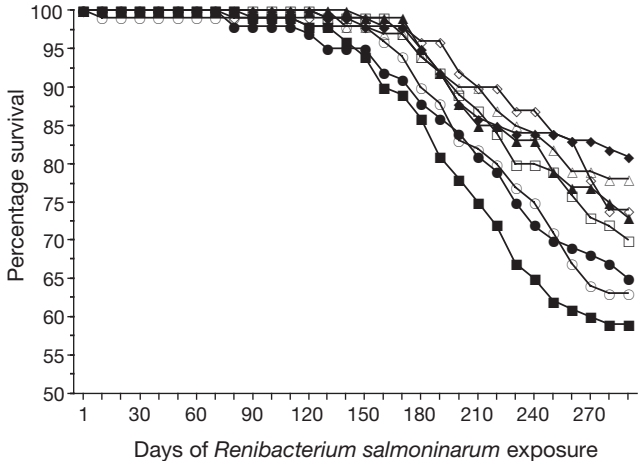

Fig. 4. Survival of experimental groups of chinook salmon during cohabitation with fish injected with *Renibacterium salmoninarum*. Injected fish were introduced into the challenge tank on Day 1. Data at 10 d intervals are shown for clarity. The experimental fish were injected with one of the following: Rs 33209 bacterin (○) , heated Rs 33209 bacterin (●), Rs MT239 bacterin (□), heated Rs MT239 bacterin (■), r-p57 emulsified with Freund's incomplete adjuvant (FIA) (△), Renogen (▲), PBS (◇) or PBS emulsified with FIA (◆)

screened for *R. salmoninarum* infection by the fluorescent antibody test (FAT). Two of the sham-injected fish were FAT positive but in each case, only 1 *R. salmoninarum* cell was observed in 50 microscope fields. In contrast, high concentrations of *R. salmoninarum* cells were observed in kidney smears from the *R. salmoninarum*-injected fish.

Among the test fish in the sham challenge tank, there were a total of 5 mortalities over the duration of the experiment; 1 fish each in the heat-treated Rs 33209 and Rs MT239 bacterin groups, as well as the PBS/FIA-injected treatment group, and 2 fish in the r-p57 group.

The percentage survival within the test groups in the *Renibacterium salmoninarum* challenge tank ranged from 59 to 81% (Fig. 4). The mean day-to-death ranged from 241 to 269 d (Table 1). There were no differences in the percentage survival among the PBS-, PBS/FIA-, r-p57- and Renogen-injected groups (Table 2). There also were no differences in survival among the bacterin groups, regardless of whether the bacterial cells had been heated or left untreated prior to injection. The survival among fish in the heated Rs MT239 group was less than that of the PBS-, PBS/FIA-, r-p57- and Renogen-injected groups. Survival in both the Rs 33209 and heated Rs 33209 groups was less than in the r-p57 and PBS/FIA groups.

DISCUSSION

We believe that the cohabitation *Renibacterium salmoninarum* challenge used in this study provided a 'real world' test of the candidate vaccines. Cohabitation challenges are unlike challenges that rely on injection or immersion with the bacterium. When bacteria are injected into the host, they circumvent a number of a fish's natural barriers to infection, including the mucus and the skin. Although immersion challenges do not allow bacteria to bypass these natural barriers, the fish are typically exposed to unnaturally

Table 1. Mortality among groups of chinook salmon vaccinated with various control or *Renibacterium salmoninarum* preparations followed by a cohabitation challenge

Treatment group	Mean day-to-death	Percentage mortality
Rs 33209	248	37
Heated Rs 33209	256	35
Rs MT239	266	30
Heated Rs MT239	241	41
Recombinant p57	264	22
Commercial vaccine	268	27
PBS	264	26
PBS/FIA	269	19

Table 2. Results of paired logrank analysis of percentage survival among groups of chinook salmon injected with various control or *Renibacterium salmoninarum* preparations followed by cohabitation with *R. salmoninarum*-injected fish

	Rs 33209	Rs 33209 HT	Rs MT239	Rs MT239 HT	rp57/FIA	Renogen	PBS/FIA
Rs 33209 HT	0.8418						
Rs MT239	0.2568	0.3735					
Rs MT239 HT	0.4188	0.3384	0.0601				
r-p57/FIA	0.0223	0.0383	0.2257	0.0027			
Renogen	0.1197	0.1879	0.6638	0.0219	0.6400		
PBS/FIA	0.0065	0.0128	0.0949	0.0007	0.6400	0.2159	
PBS	0.0627	0.1126	0.4763	0.0100	0.5990	0.7943	0.3062

high concentrations of the pathogen to ensure infection. In both injection and immersion challenges, the fish are also subjected to the additional stress of handling. Furthermore, to obtain the needed numbers of bacteria for an injection or immersion challenge the bacteria are grown *in vitro*, which in some cases can cause attenuation of some bacterial pathogens. The cohabitation challenge not only eliminates the technical difficulties of injection and immersion challenges, but also best approximates the natural interaction of an uncompromised host and a virulent pathogen. A vaccine that provides a slight immunological advantage to the host should have a measurable effect in a challenge that does not force the fish to suddenly interact with unnaturally high numbers of bacteria.

The results of this study highlight one of the difficulties of a cohabitation challenge, where the amount of time required to establish lethal levels of *Renibacterium salmoninarum* in the test fish can be significantly longer than by injection or immersion challenges (Sanders et al. 1978, Beacham & Evelyn 1992, Murray et al. 1992). The amount of time between the introduction of the infected fish and the onset of mortality among the test fish in the *R. salmoninarum* challenge tank was expected to be about 2 mo. The mortality rates were expected to increase in such a manner that about 4 mo after introduction of the livebox fish the experiment could be terminated with mortality among the PBS-vaccinated fish exceeding 50%. Although the deaths among the test fish did begin about 2 mo after introduction of the livebox fish, the mortality did not increase as expected and we did not attain 50% mortality in any of the groups. Unfortunately, we could only monitor the fish for 285 d due to constraints of wet-laboratory space and scheduling of other experiments.

The concentration of *Renibacterium salmoninarum* in the rearing water was determined using the MF-FAT (McKibben & Pascho 1999) that does not differentiate live from dead cells. *R. salmoninarum* cells were detected in water samples from both the sham and challenge tanks after introduction of the livebox fish, but probably for different reasons. In the *R. salmoninarum* challenge tank, the bacterial load reached about 10 000 cells ml^{-1} on Day 38 and remained steady for the duration of the experiment. These cells probably represented live bacteria shed both from the *R. salmoninarum*-injected fish placed in the live boxes and (later) from infected test fish. The cells detected in the sham tank water samples may have been formalin-inactivated cells that were shed from the test fish vaccinated with whole cell *R. salmoninarum* bacterins. After 150 d, the bacteria were no longer detected in water from the sham tank, suggesting that the fish had stopped shedding the bacterin cells and that there were no active *R. salmoninarum* infections. Although it seems unlikely, because of the high survival rate in the sham challenge tank, the *R. salmoninarum* cells detected in that tank may have been live bacteria shed into the water from a low number of infected fish. However, the concentration of bacteria was not great enough to perpetuate an epizootic in the sham challenge tank and bacteria were no longer detected 150 d after introduction of the livebox fish. If bacterin cells were also shed from the vaccinated test fish in the *R. salmoninarum* challenge tank, they probably made up only about 0.1% of the total number of cells counted. These results indicated that the livebox fish in the *R. salmoninarum* challenge tank were shedding *R. salmoninarum* at concentrations great enough to infect healthy fish residing in the same water system (Elliott & Pascho 1995).

In this study, we examined the efficacy of 5 potential *Renibacterium salmoninarum* vaccines that differed relative to the amount of p57. The recombinant form of p57 allowed use of a virtually pure form of the protein that is known to elicit a strong antibody response in several salmonid species (Alcorn et al. 2002, Alcorn & Pascho 2002). Both the Rs 33209 and Rs MT239 genomes contain 2 copies of the p57 gene (O'Farrell & Strom 1999), and the p57 protein is produced by both strains. However, the protein does not associate with the Rs MT239 cell surface (Bruno 1990, Senson & Stevenson 1999), so only p57 proteins within the Rs MT239 cells would be present in the untreated vaccine. Complete removal of internal and cell surface p57 from Rs 33209, and internal p57 from Rs MT239, was done according to established methods (Piganelli et al. 1999a) and should have exposed antigens normally masked by the p57 (Wood & Kaattari 1996). The *R. salmoninarum* vaccines used in this study, therefore, provided a range of p57 exposure to the chinook salmon immune system.

Unfortunately, none of the vaccines tested in the current study conferred protection to the chinook salmon during an *Renibacterium salmoninarum* cohabitation challenge. Based on our understanding of the disease process during an *R. salmoninarum* infection (Kaattari & Piganelli 1997), increased mortalities were expected among the fish injected with r-p57. The amount of r-p57 protein injected into the fish typically induces a measurable antibody response (Alcorn & Pascho 2002, Alcorn et al. 2002). If a strong antibody response to the r-p57 occurs, then the subsequent exposure of the fish to p57 from an *R. salmoninarum* infection would be expected to increase the glomerulonephritis associated with BKD. The lack of increased mortalities among the r-p57-injected fish may indicate that an antibody response was not induced, or that the antibodies did not recognize the native p57 protein. However, based

on our previous antibody response studies (Alcorn & Pascho 2002, Alcorn et al. 2002), we believe that the amount of r-p57 injected did induce an antibody response. Also, during studies conducted to determine the bacterin dose, it was observed that 50 µg of r-p57 induced a strong antibody response (Fig. 2). The monoclonal antibodes, 3H1 and 4D3, recognized r-p57 (Fig. 1) and fish exposed to the native p57 protein produced antibodies that recognized the r-p57 in an ELISA (Fig. 2). These results indicate a similar structure of recombinant and native p57. Therefore, the biological implications of injecting fish with the r-p57 remain unclear, because the protein neither provided protection nor exacerbated mortality under the conditions of the current study.

Whereas native p57 is considered to be an immunodominant protein of *Renibacterium salmoninarum* (Bartholomew et al. 1991), its suitability as a vaccine remains questionable. We reported previously that both chinook salmon and rainbow trout will produce a measurable antibody response to the native p57 (Alcorn & Pascho 2002). In the current study, however, any antibody response may have been inadequate, as the r-p57 and p57(+) whole-cell *R. salmoninarum* bacterins failed to elicit protective immunity. Recently, Rhodes et al. (2004) tested the efficacy of both live and formalin-inactivated Rs MT239 vaccines for post-smoltification chinook salmon by challenging the fish by IP injection of live Rs 33209. The limited protection that Rhodes et al. (2004) described is in contrast to the results of the current study. The differences in protection may have been due to a number of factors including the stock of fish and their life stage. Also, Rhodes et al. (2004) used a bivalent *Vibrio* vaccine prior to seawater transfer, a prime-boost vaccination strategy for the MT239 preparations, an IP injection of *R. salmoninarum* for the challenge, and a chinook salmon population which was already infected with *R. salmoninarum*.

It has been speculated that removal of the *Renibacterium salmoninaum*-associated p57 might expose other, more protective antigens on the cell surface (Wood & Kaattari 1996). Piganelli et al. (1999b) reported that vaccination of coho salmon *Oncorhynchus kisutch* with the *R. salmoninarum* cells that were heat-treated in a manner similar to that used in the current study (Piganelli et al. 1999a) increased their survival when compared to unvaccinated fish. Conversely, the heat-treated (p57-removed) Rs MT239 cells actually exacerbated mortality in the current study, as evidenced by a comparatively earlier onset of mortality among fish in that group, and consequently, a shorter mean-day-to-death compared to the other groups. Our contrasting results with the p57-removed bacterins may simply demonstrate how slight differences in the manner in which fish are vaccinated and challenged can affect the outcome of a vaccine trial. Both the route of immunization (Kaattari & Piganelli 1996) and the inclusion of an adjuvant in the delivery of a vaccine (Anderson 1992) can affect the antibody response and disease resistance of fish. Piganelli et al. (1999b) used injection and immersion *R. salmoninarum* challenges to measure the efficacy of the p57-removed *R. salmoninarum* vaccine delivered as either an intraperitoneal and intramuscular injected vaccine emulsified in the adjuvant FIA, or as an oral vaccine. In contrast, we suspended the *R. salmoninarum* bacterins in PBS for injection and then used a longer, more natural, cohabitation challenge.

The observed efficacy of a *Renibacterium salmoninarum* vaccine also may be affected by the relationship between the fish's immune response to the vaccine and the subsequent response to the challenge bacteria. For example, the results shown in Fig. 2 suggested that the humoral response to an *R. salmoninarum* bacterin was concentration-dependent and may not reach its peak for about 9 wk after injection. Since Piganelli et al. (1999b) used an injection challenge, the fish did not have time to develop an antibody response to the p57 before they died. The non-p57 antibodies that they produced against the p57-removed bacterin did provide some protection. In contrast, when the fish were challenged by cohabitation in our study, the fish had a greater length of time before the infection overwhelmed them. During that time, they could produce an antibody response to the p57 in addition to a secondary response to any available non-p57 antigens. Thus, increased amounts of antigen-antibody complexes would more quickly cause the development of BKD kidney lesions leading to earlier mortality. If this hypothesized scenario is correct, the use of bacterins based on p57-removed *R. salmoninarum* cells may be ineffective because fish reared in a hatchery environment may be responding to *R. salmoninarum* as a consequence of their exposure to bacteria shed from infected cohorts.

We also tested a commercial live-cell vaccine of *Arthrobacter* sp. nov., a species which shares common antigenic determinants to *Renibacterium salmoninarum* according to the manufacturer. For optimal protection, the fish needed to be at least 10 g at the time of immunization. A protective immune response would then develop over the course of 400 degree-days (days after immunization × water temperature [°C]). The average weight of the fish was 9.1 g 52 d prior to the time of immunization (to reduce stress, the fish weights were not determined after they had reached a target weight of 9 g at a normal growth rate). Since the water temperature remained at 12°C throughout our study, a protective immune response should have developed at

about 33 d. The concentration of *R. salmoninarum* in the challenge tank water increased significantly between 25 and 38 d after addition of the livebox fish, or 39 to 52 d post immunization (Fig. 3). The commercial vaccine did not produce a protective immune response in chinook salmon in this cohabitation challenge. Renogen has been reported by the manufacturer to be protective for Atlantic salmon injected intraperitoneally with *R. salmoninarum*. The differences in the results may be due to the host species, the challenge method or other hereunto unexplained parameters. Limited protection to BKD of chinook salmon vaccinated with Renogen after smoltification has also been described (Rhodes et al. 2004). The differences in Renogen efficacy between the current study and that of Rhodes et al. (2004) may have been due to the variations in the studies described previously.

In summary, this work suggests that chinook salmon were not protected from BKD when injected with any of the vaccines against *Renibacterium salmoninarum*. Due to the ability of *R. salmoninarum* to survive and even replicate within host macrophages (Bandin et al. 1993, Gutenberger et al. 1997), and the detrimental consequences of inducing an antibody response, future research should focus on stimulation of the cellular immune response. Activated cytotoxic T lymphocytes (Nakanishi et al. 2002) and nonspecific cytotoxic cells could kill host cells harboring the bacteria and also secrete interleukins to activate macrophage cells (Graham & Secombes 1988, Neumann et al. 1995). Activated fish macrophages have been shown to have increased ability to kill *R. salmoninarum* (Hardie et al. 1996) and should also stimulate the cytotoxic cells.

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