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Mortality and kidney histopathology of chinook salmon *Oncorhynchus tshawytscha* exposed to virulent and attenuated *Renibacterium salmoninarum* strains

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ABSTRACT: An isolate of *Renibacterium salmoninarum* (strain MT 239) exhibiting reduced virulence in rainbow trout *Oncorhynchus mykiss* was tested for its ability to cause bacterial kidney disease (BKD) in chinook salmon *Oncorhynchus tshawytscha*, a salmonid species more susceptible to BKD. Juvenile chinook salmon were exposed to either 33209, the American Type Culture Collection type strain of *R. salmoninarum*, or to MT 239, by an intraperitoneal injection of 1×10^3 or 1×10^6 bacteria $fish^{-1}$, or by a 24 h immersion in 1×10^5 or 1×10^7 bacteria ml^{-1}. For 22 wk fish were held in 12°C water and monitored for mortality. Fish were sampled periodically for histological examination of kidney tissues. In contrast to fish exposed to the high dose of strain 33209 by either injection or immersion, none of the fish exposed to strain MT 239 by either route exhibited gross clinical signs or histopathological changes indicative of BKD. However, the MT 239 strain was detected by the direct fluorescent antibody technique in 4 fish that died up to 11 wk after the injection challenge and in 5 fish that died up to 20 wk after the immersion challenge. Viable MT 239 was isolated in culture from 3 fish that died up to 13 wk after the immersion challenge. Total mortality in groups injected with the high dose of strain MT 239 (12%) was also significantly lower ($p < 0.05$) than mortality in groups injected with strain 33209 (73%). These data indicate that the attenuated virulence observed with MT 239 in rainbow trout also occurs in a salmonid species highly susceptible to BKD. The reasons for the attenuated virulence of MT 239 were not determined but may be related to the reduced levels of the putative virulence protein p57 associated with this strain.

KEY WORDS: *Renibacterium salmoninarum* · Attenuated strain · Pathogenesis · p57 · Histopathology · BKD

INTRODUCTION

Bacterial kidney disease (BKD), caused by the gram-positive bacterium *Renibacterium salmoninarum*, continues to be a serious problem affecting salmonid fishes worldwide (Fryer & Lannan 1993). A major impediment to the development of successful methods of BKD control is the lack of understanding regarding *R. salmoninarum* pathogenesis (Evenden et al. 1993). Because methods for the genetic manipulation of *R. salmoninarum* (e.g., gene knockout procedures) have not yet been optimized for use with this slow-growing bacterium, the study of attenuated strains provides one method for examining the virulence mechanisms of this pathogen.

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Renibacterium salmoninarum isolates from different geographic locations have been analyzed to compare their antigenic determinants, biochemical properties, genetic diversity, and virulence. In general, investigators have shown strong similarities among isolates (Getchell et al. 1985, Bruno & Munro 1986, Fiedler & Draxl 1986, Daly & Stevenson 1987, 1990, Radacovici & Dubreuil 1991, Starliper 1996). Some differences have been detected, however, based on *in vitro* analyses (Austin & Rodgers 1980, Arakawa et al. 1987, Bandín et al. 1992, Farias et al. 1994) and an *in vivo* virulence comparison (Starliper et al. 1997).

Three non-agglutinating strains of *Renibacterium salmoninarum* (MT 239, MT 240, and MT 241) have been identified and lower BKD-related mortality was attributed to these strains in comparison to agglutinating strains following an injection challenge of rainbow trout *Oncorhynchus mykiss* (Bruno 1988). Fish exposed to the strain MT 239 had the lowest mortality (8%), and the reduced virulence reported with this strain in rainbow trout was also observed in a later study by Senson & Stevenson (1999). The MT 239 strain produces a 57 kDa protein (p57) that has been associated with autoagglutination and virulence (Bruno 1988, 1990), but it differs from the virulent strains, including the type strain 33209 of the American Type Culture Collection (33209), in having no detectable p57 associated with its cell surface (Bruno 1990, Daly & Stevenson 1990, Wood et al. 1995). In addition, analysis of MT 239 by electron microscopy has failed to demonstrate the amorphous layer surrounding the bacterium (Senson & Stevenson 1999) that is observed with virulent, agglutinating strains and is believed to partially consist of p57 (Dubreuil et al. 1990, Senson & Stevenson 1999). Recently, it was shown that low levels of p57 are associated with whole cell preparations of strain MT 239, but these levels are much lower compared to virulent strains (O'Farrell & Strom 1999, Senson & Stevenson 1999). In contrast, comparable levels of p57 are found in the culture supernatants (O'Farrell & Strom 1999, Senson & Stevenson 1999). Genetic analysis revealed that the sequence of *msa* in MT 239 is identical to the *msa* sequence in 33209, and the expression of *msa* is nearly equivalent in the 2 strains (O'Farrell & Strom 1999).

Because few studies have been conducted with the non-agglutinating MT 239 strain, the cause of its attenuated virulence has not been determined. Moreover, it is not known whether the attenuated virulence of MT 239 in rainbow trout extends to other salmonids that have a much higher susceptibility to BKD (Sanders et al. 1978, Bell 1987). High morbidity caused by *Renibacterium salmoninarum* is prevalent in populations of Pacific salmon, including chinook salmon *Oncorhynchus tshawytscha* (Fryer & Lannan 1993). To understand further the basis for the attenuation of MT 239 and to determine whether the attenuated virulence previously reported in rainbow trout also occurs in a species that is highly susceptible to BKD, the virulence and histopathological changes associated with the virulent strain 33209 and the attenuated strain MT 239 were observed in chinook salmon. The onset and progression of BKD, morbidity, and mortality were compared between groups of fish exposed to either *R. salmoninarum* strain by an injection challenge or a separate immersion challenge.

MATERIALS AND METHODS

Experimental fish. Eggs from brood year 1996 chinook salmon *Oncorhynchus tshawytscha* of the Yaquina River stock (Oregon, USA) served as the source of experimental fish for the study. These eggs were pooled from 8 mating pairs in which both parents had undetectable or very low levels of *Renibacterium salmoninarum* on the basis of testing kidney tissue (males) or testing both kidney tissue and ovarian fluid (females) by the enzyme-linked immunosorbent assay (ELISA; Pascho et al. 1991). The eyed eggs were transferred to the Western Fisheries Research Center, Biological Resources Division of the United States Geological Survey, Seattle, Washington, USA, where the study was conducted.

Prior to bacterial challenge, experimental fish (about 1 yr old) were weighed (approximately 12.5 g), and kidney and spleen sampled from 60 fish and processed for ELISA following the procedure described by Pascho et al. (1991). Of the 60 fish, 1 did not provide enough tissue for ELISA testing, 19% tested negative, and the remaining 80% tested positive for *Renibacterium salmoninarum* antigen, but all of those testing positive had values corresponding to low antigen levels (Pascho et al. 1991, Elliott et al. 1997). It was not determined whether the ELISA-positive fish were infected with viable *R. salmoninarum* or merely carried persistent antigen (Pascho et al. 1997). Twelve of these fish were also tested for the presence of *R. salmoninarum* by the direct fluorescent antibody technique (DFAT) (Pascho et al. 1987). Briefly, the entire kidney and spleen were pooled from individual fish, homogenized without dilution, and used to prepare duplicate tissue smears for DFAT. The smears were tested with goat anti-*R. salmoninarum* polyclonal antiserum (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) at a 1:40 (v/v) dilution in 0.01 M phosphate-buffered saline (PBS), pH 7.2. One hundred microscope fields at 1000× magnification were examined per slide, and the number of *R. salmoninarum* cells per 100 fields was recorded. None of the fish tested by the DFAT were positive for *R. salmoninarum*.

During the experiment, the fish were maintained in either 28 l (for histology sampling) or 137 l (for mortality monitoring) circular tanks in 12°C, sand-filtered, UV-treated, Lake Washington water, and each tank contained 30 fish. Fish were fed BioOregon pellets (Bioproducts, Inc., Warrenton, OR, USA) at an amount equivalent to 1.5% of body weight every other day. Feed was withheld 24 h before injection and immersion challenges, during the 24 h immersion challenge, and 24 h prior to histological sampling. Feeding resumed 48 h post-challenge.

Bacterial strains and growth conditions. The 2 *Renibacterium salmoninarum* strains used in this study were the type strain 33209 (American Type Culture Collection, Manassas, VA, USA), originally isolated from a chinook salmon in Oregon, and strain MT 239, originally isolated from Atlantic salmon in Scotland and selected for avirulence (Bruno 1988). Both *R. salmoninarum* strains were grown at 15°C with constant stirring in KDM2 broth (Evelyn 1977), which also included 0.05% (w/v) cysteine-HCl and 10% (v/v) fetal bovine serum (FBS).

Growing cultures of *Renibacterium salmoninarum* were harvested after 9 d by centrifuging at 4923 × *g* at 4°C for 20 min. The pellets were resuspended in 35 ml PBS (0.01 M, pH 7.4) supplemented with 0.1% (w/v) peptone (PBS-peptone). The concentrations of bacteria in the broth cultures were determined by a membrane filtration-fluorescent antibody staining procedure (Elliott & Barila 1987) modified for use with 4′,6-diamidino-2-phenylindole (DAPI) stain (Sigma, St. Louis, MO, USA). The modified procedure used 100 µl of DAPI stain at 1 µg ml^{-1} with PBS (0.01 M, pH 7.2) in place of the fluorescein-labeled goat-anti-*R. salmoninarum* antibody. The concentrated *R. salmoninarum* solutions were diluted in PBS-peptone to prepare the challenge suspensions: 1×10^3 cells $fish^{-1}$ (low-dose injection), 1×10^6 cells $fish^{-1}$ (high-dose injection), 1×10^5 cells ml^{-1} (low-dose immersion), and 1×10^7 cells ml^{-1} (high-dose immersion). To verify culture viability, 10-fold dilutions of each low-dose bacterial suspension were inoculated onto KDM2 agar plates and incubated at 15°C for 6 wk.

Challenge procedure. For the injection challenge, fish were anesthetized with MS 222 (tricaine methanesulfonate, Argent Chemical Laboratories, Redmond, WA, USA) and then injected intraperitoneally with 100 µl of either PBS-peptone (as a control) or the low or high injection doses of *Renibacterium salmoninarum*. Thirty fish were placed in each tank; 3 tanks for each dilution were used for each of the 2 bacterial strains. Two of the 3 tanks (137 l) were used for monitoring mortality, and the third tank (28 l) was used for histological sampling. Thus, 90 fish were used per treatment for each bacterial strain.

For the immersion challenge, 30 fish were placed in each challenge bucket with 15 l of sand-filtered, UV-treated, Lake Washington water and either 150 ml of the control PBS-peptone solution or equivalent volumes of the low or high immersion dose of *Renibacterium salmoninarum*. Three replicates were used for each dilution per *R. salmoninarum* strain, with 2 buckets for monitoring mortality and 1 for histological examination. The immersion challenge was done in a static system for 24 h. The temperature was maintained at 12°C by placing the buckets in 1.22 m diameter circular tanks partly filled with continuously flowing water. Oxygen was continuously supplied to the individual buckets during the challenge. Following the challenge, the fish were transferred either to the 28 l (histological sampling) or the 137 l tanks (monitoring mortality).

Bacterial culture, FAT, and PCR. Throughout the experiment, fish that died or were removed for histological analysis were examined for signs associated with BKD including the presence of lesions affecting the kidney, spleen and swim bladder. Kidney-spleen homogenates from fish that died were analyzed for the presence of *Renibacterium salmoninarum* by DFAT. Tissue from the anterior kidney of fish that died was inoculated onto selective KDM2 agar plates (Austin et al. 1983). Culture plates were incubated at 15°C and examined after 6 to 8 wk for colonies characteristic of *R. salmoninarum*. Smears from selected colonies were tested by DFAT.

Representative colonies isolated from fish that died in challenges with 33209 and MT 239 were tested by the polymerase chain reaction (PCR) for further confirmation of their identity as *Renibacterium salmoninarum*. Methods for PCR followed those previously described by Pascho et al. (1998) for first round amplification with exceptions as noted. Briefly, the primers used were derived from the *R. salmoninarum* sequence of *msa* (GenBank accession number Z12174) and were forward 1014-1031 (5′-ACTGGTAAATGGTGGTCT-3′; A8) and reverse 1302-1319 (5′-ACCAATCGATGTTTTACC-3′; B6), which amplified a 305 base pair product. The 25 µl reaction mixture included 2.5 µl Taq reaction buffer, 1.5 mM $MgCl_2$, 1 µM of each primer, 0.2 mM of each nucleotide, and 0.625 U Taq polymerase. Amplification conditions were 35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min, followed by 1 cycle of 94°C for 1 min, 50°C for 1 min, and 72°C for 10 min. Bacterial colonies isolated in culture were boiled in 25 µl of H_2O for 5 to 7 min, and 5 µl of this mixture were used in the PCR.

All of the fish that were still alive 22 wk after challenge were euthanized by an overdose of MS 222, and immediately frozen. Of these fish, 5 from each replicate tank of the control injection and immersion, the high-dose MT 239 injection and immersion, and the

high-dose 33209 injection and immersion groups were processed for DFAT of kidney-spleen homogenates.

Histological examination. Three fish were collected for histological analysis at 1, 6, and 10 wk post-injection, and at 8 wk post-immersion from the control and high-dose histology sampling tanks. At the conclusion of the experiment (22 wk), 2 fish were also sampled for histological examination from each control and high-dose mortality monitoring tank from both the injection and immersion challenges. Fish were overdosed with MS 222 and preserved in Poly/LEM buffered formaldehyde fixative (Carson et al. 1973). The kidneys were later removed and 5 µm sections prepared for staining with haematoxylin and eosin.

The indirect fluorescent antibody technique (IFAT) was used to identify *Renibacterium salmoninarum* in tissue sections (procedure for paraffin sections modified from Bullock et al. 1980, Pascho et al. 1987). The IFAT protocol included deparaffinization of the section followed by an incubation with a protease solution (bacterial protease type VIII, Sigma; diluted 1 mg ml^{-1} in 0.01 M PBS, pH 7.5) for 1 h at room temperature in a humid chamber. Samples were then rinsed 3 times (5 min each) with 0.01 M PBS, pH 7.2. The primary antibody, pooled sera from 2 rabbits immunized with killed whole cells of MT 239, was diluted 1:40 (v/v) in PBS (pH 7.2) and added to sections for 1 h at room temperature. The antibody was previously tested with cultures of both 33209 and MT 239 and reacted strongly with both strains. Slides were rinsed 3 times with PBS, then incubated for 1 h at room temperature with FITC-conjugated goat anti-rabbit antibody (Sigma) diluted 1:500 (v/v) in 0.01 M PBS, pH 7.2. The remaining steps included a 5 min rinse with PBS, a 1 min incubation with the Eriochrome Black-T counterstain diluted 1:60 (w/v) in PBS, a brief rinse with PBS, and a 5 min rinse in PBS. The IFAT samples were mounted on glass slides with glycerol-based DABCO mounting medium (Johnson et al. 1982).

For the histological analysis of fish at 22 wk post-challenge, the entire kidney of each sampled fish was divided longitudinally into 2 approximately equal-sized halves. One piece was processed for histological examination as previously described. The remaining kidney and the entire spleen from each of these fish were processed for analysis by DFAT. Material from the kidney-spleen homogenate from 1 fish in each control and high-dose treatment of the injection and immersion challenges (6 fish total) was inoculated onto KDM2 agar plates for *Renibacterium salmoninarum* isolation.

Statistical analysis. To compare total mortality between treatments within each challenge (immersion or injection) at 22 wk following the challenges, an arcsine transformation was used for the percent mortality data from each tank. The transformed data were then analyzed by a 1-factor analysis of variance (ANOVA) and Scheffé's *F* multiple comparison post-hoc test to compare mortality in the control and treatment groups for a given bacterial strain. A 2-factor ANOVA was used to compare the effects of the bacterial strain and *Renibacterium salmoninarum* dose on mortality, and to determine interactive effects of dose and strain.

RESULTS

Mortality

Injection challenge

Although the first death occurred 12 d after challenge, the first death attributed to BKD occurred 40 d post-challenge in a fish injected with the high dose of 33209. By 22 wk following the challenge, 73% of the fish injected with the high dose of 33209 had died compared to 12% of fish injected with the same dose of MT 239 (Fig. 1). Total mortality differed between the control and the 33209 treatments ($p = 0.003$; Table 1). Among the 33209 challenge groups, mortality was greater in the high-dose group compared to both the control ($p = 0.004$) and the low-dose groups ($p = 0.004$), but no difference in mortality was observed between the low-dose and the control groups ($p > 0.05$). No dif-

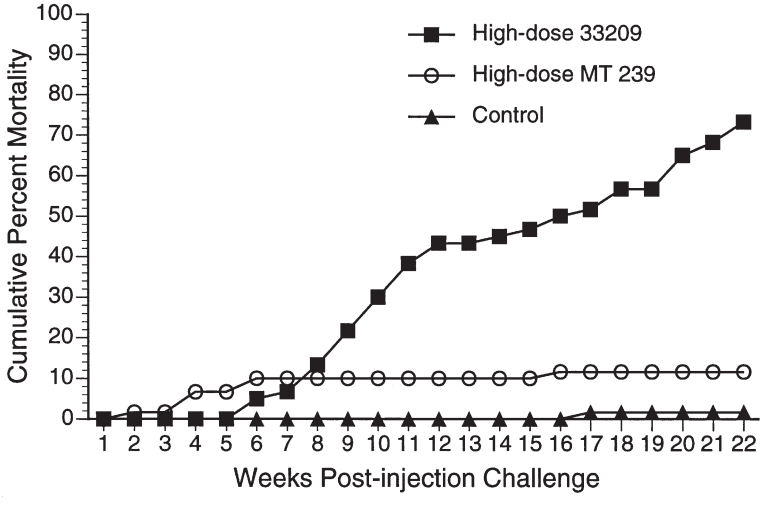

Fig. 1. Mean cumulative percent mortality of chinook salmon *Oncorhynchus tshawytscha* injected with either a high dose of 33209 or MT 239 strains of *Renibacterium salmoninarum* or a control PBS solution. Fish were monitored for 22 wk at 12°C

Table 1. *Oncorhynchus tshawytscha.* Total mortality (22 wk after challenge) of juvenile chinook salmon challenged by injection or immersion with 2 doses of *Renibacterium salmoninarum* strain MT 239 or 33209

Challenge type and bacterial strain	Dose[a] and replicate	No. dead/total (%)	No. of deaths attributed to BKD[b]/total dead
Injection			
Control	1	0/30 (0)	0/0
	2	1/30 (3)	0/1
MT 239	Low dose-1	2/30 (7)	0/2
	2	1/30 (3)	0/1
	High dose-1	3/30 (10)	0/3
	2	4/30 (13)	0/4
33209	Low dose-1	1/29 (3)	0/1
	2	0/29 (0)	0/1
	High dose-1	24/30 (80)	22/24
	2	20/30 (67)	20/20
Immersion			
Control	1	4/30 (13)	0/4
	2	2/30 (7)	0/2
MT 239	Low dose-1	1/29 (3)	0/1
	2	5/29 (17)	0/5
	High dose-1	2/29 (7)	0/2
	2	2/30 (7)	0/2
33209	Low dose-1	1/30 (3)	No sample
	2	9/30 (30)	1/9
	High dose-1	4/30 (13)	3/4
	2	18/30 (60)	15/18

[a]Challenge doses: low dose injection 1×10^3 bacteria fish^{-1}; high dose injection 1×10^6 bacteria fish^{-1}, low dose immersion 1×10^5 bacteria ml^{-1}; high dose immersion 1×10^7 bacteria ml^{-1}

[b]Fish classified as dying from BKD had been tested by DFAT and had cell numbers of *R. salmoninarum* too numerous to count in each microscopic field

ference in mortality was detected between the control and MT 239 groups ($p > 0.05$). Analyses for strain and dose effects showed higher mortality in the 33209 treatment groups compared to the MT 239 treatment groups ($p = 0.004$). These analyses also showed that the *Renibacterium salmoninarum* challenge dose of either strain had an effect on mortality ($p = 0.0005$) and that there was an interactive effect between the *R. salmoninarum* strain and dose ($p = 0.002$). The single control fish that died, the few fish that died in the low-dose injection groups, and all but 1 of the fish that died in the high-dose MT 239 group all showed severe erosion of the caudal fin. The caudal lesions were associated with unidentified gram-negative rods and closely resembled coldwater disease caused by *Flavobacterium psychrophilum* (hereafter referred to as a *Flavobacterium*-like infection; Table 2).

Immersion challenge

Mortality was variable between the replicate tanks (Table 1), and some tanks had a higher prevalence of a

Table 2. *Oncorhynchus tshawytscha.* Results of detection of *Renibacterium salmoninarum* by bacterial culture and DFAT in kidney-spleen homogenates from fish that died in injection and immersion challenges with 2 strains of the bacterium (MT 239 and 33209)

Challenge type and treatment group[a]	*R. salmoninarum* culture results: no. positive/ total sampled	DFAT detection of *R. salmoninarum* No. positive/ total tested	DFAT detection of *R. salmoninarum* No. highly infected[b]/ total tested	No. with *Flavobacterium*-like infection/total dead
Injection				
Control	0/1	0/1	0/1	1/1
Low-dose MT 239	0/2	2/3[c]	0/3	3/3
High-dose MT 239	0/1	2/7	0/7	6/7
Low-dose 33209	0/0	1/1[c]	0/1	1/1
High-dose 33209	35/35	44/44	42/44	11/44
Immersion				
Control	0/2	1/6[c]	0/6	5/6
Low-dose MT 239	1/4	3/6	0/6	5/6
High-dose MT 239	2/3	2/4[c]	0/4	3/4
Low-dose 33209	2/4	3/9	1/9	8/10
High-dose 33209	16/16	22/22	18/22	18/22

[a]Refer to Table 1 for bacterial concentrations of the treatment doses

[b]Fish classified as being highly infected had been tested by DFAT and had cell numbers of *R. salmoninarum* too numerous to count in each microscopic field

[c]Low numbers of bacteria per 100 microscopic fields (<10 *R. salmoninarum* cells)

Flavobacterium-like infection (Table 2). No difference in mortality was observed between the control and the 33209 groups or between the control and MT 239 groups ($p > 0.05$). Comparisons of the effect on mortality of the *Renibacterium salmoninarum* strain (33209 or MT 239), the dose of *R. salmoninarum*, and the interactive effect of strain and dose, detected no differences ($p > 0.05$).

Bacterial culture, FAT and PCR

The bacteria that were re-isolated in culture from the MT 239-challenged fish appeared faint in fluorescence intensity compared with bacteria re-isolated from the 33209 fish when tested by the DFAT. Nevertheless, all of the isolates suspected to be *Renibacterium salmoninarum* from the MT#239 fish displayed appropriate colony and cell morphology for *R. salmoninarum*, and their identity was confirmed by PCR testing. Previous testing of the MT 239 strain by the DFAT showed that these cells stain poorly with the fluorescein-labeled goat-anti-*R. salmoninarum* antibody compared with strains that produce large amounts of surface-associated p57.

Injection challenge

Of the fish that died in the high-dose 33209 group and tested by bacterial culture, all showed characteristic *Renibacterium salmoninarum* growing on selective KDM2 agar plates (Table 2). The colonies were confirmed to be *R. salmoninarum* by DFAT, and the identity of a representative isolate was further confirmed by PCR. In samples from fish that died in the low-dose and high-dose MT 239 groups, there was no growth of *R. salmoninarum* on KDM2 agar (Table 2). Likewise, *R. salmoninarum* was not isolated from fish that died in the control group (Table 2).

In the high-dose 33209 group, all of the fish that died tested positive for *Renibacterium salmoninarum* by the DFAT (Table 2). Of these, 95% were highly infected with *R. salmoninarum*, thus indicating that these fish died of BKD (Tables 1 & 2). Of the fish that died in the high-dose MT 239 group, only 2 tested positive for *R. salmoninarum* by DFAT (Table 2), and the bacteria observed were faintly fluorescent, which suggested that they were MT 239. *R. salmoninarum* was identified by the DFAT in fish that died in the MT 239 exposure up to 11 wk post-challenge. Of 10 surviving fish sampled from the control and high-dose groups at the end of the experiment, 20% tested positive for *R. salmoninarum* by DFAT in the control group and 60% in the high-dose 33209 group were positive (Table 3).

Table 3. *Oncorhynchus tshawytscha.* Detection of *Renibacterium salmoninarum* by DFAT in kidney-spleen homogenates from surviving fish tested 22 wk after challenge by injection or immersion with strain MT 239 or 33209

Challenge type and treatment group[a]	No. positive for *R. salmoninarum* in each group tested[b]
Injection	
Control	2
High-dose MT 239	0
High-dose 33209	6[c]
Immersion	
Control	0
High-dose MT 239	0
High-dose 33209	4[d]

[a]Refer to Table 1 for bacterial concentrations of the treatment doses
[b]10 fish were tested in each of the control and high-dose treatment groups
[c]The numbers of *R. salmoninarum* cells/100 microscopic fields were 2, 3, 6, 9, 17, and 447. None were too numerous to count
[d]The numbers of *R. salmoninarum* cells/100 microscopic fields were 12, 18, 37, and 1 sample had *R. salmoninarum* too numerous to count in each microscopic field

Of all the fish that tested positive, only 1 fish from the high-dose 33209 group had a high number of *R. salmoninarum* cells. None of the fish tested at the end of the experiment from the high-dose MT 239 group tested positive for *R. salmoninarum* (Table 3).

Immersion challenge

Despite the occurrence of a *Flavobacterium*-like infection, *Renibacterium salmoninarum* was re-isolated by bacterial culture from some fish that died in the low-dose and from all of the dead fish tested in the high-dose 33209 groups (Table 2). *R. salmoninarum* was also recovered from some of the fish that died in the low-dose and high-dose MT 239 groups for up to 13 wk following the challenge (Table 2). *R. salmoninarum* was not recovered from fish that died in the control group; all but 1 of these fish showed evidence of a *Flavobacterium*-like infection (Table 2).

Among the fish that died in the high-dose MT 239 group, 50% tested positive for *Renibacterium salmoninarum* by the DFAT, but these fish had fewer than 5 cells per 100 microscope fields and showed evidence of a *Flavobacterium*-like infection (Table 2). *R. salmoninarum* was identified by the DFAT up to 20 wk post-challenge in some fish exposed to MT 239. The high-dose 33209 group had numerous cases of *Flavobacterium*-like infections which may have contributed to the high variability in mortality between the replicate tanks. In this treatment group, all of the fish that

died tested positive for *R. salmoninarum* by the DFAT, and most showed evidence of a *Flavobacterium*-like infection. Also, nearly all were so highly infected with *R. salmoninarum* that the numbers of cells per microscope field were too numerous to count, indicating BKD-related mortality (Tables 1 & 2). Of 10 surviving fish sampled from each group at the end of the experiment, 40% tested positive for *R. salmoninarum* by DFAT in the high-dose 33209 group, and none tested positive in the control and high-dose MT 239 groups (Table 3).

Clinical observations

Fish from the control and MT 239 groups did not exhibit external or internal signs characteristic of BKD in either the injection or immersion experiments. Clinical signs of BKD were observed, however, in fish exposed to the high doses of the 33209 strain in both the injection and immersion challenges. These signs included distended abdomen, ascites, exophthalmos, superficial blebs and abscesses on the flanks, internal petechial haemorrhaging, dark coloration, splenomegaly and white granulomatous lesions on the kidney and swim bladder.

Histological examination

Injection challenge

Fish sampled at 1, 6, 10, and 22 wk post-injection from the control and high-dose MT 239 groups did not exhibit granulomas or other histological changes in kidney tissue characteristic of a *Renibacterium salmoninarum* infection (Table 4). Focal to diffuse granulomatous tissue was observed in the kidney of fish injected with 33209 (Table 4). The numbers and types of granulomas varied from fish to fish within each sampling date, but lesions were observed in all fish sampled from the high-dose 33209 group at 10 and 22 wk. Granulomas were randomly distributed in the haematopoietic tissue of both the anterior and the posterior kidney. Degeneration and necrosis of cells in the haematopoietic tissue was evident, and many melanin granules from melanomacrophage aggregates were dispersed throughout the lesions. Principal changes in renal tissue in the lesion areas included epithelial degeneration of proximal and distal tubules, periglomerular fibrosis, hypercellularity of glomeruli resulting in the occlusion of Bowman's space, and thickening of the basement membrane of Bowman's capsule.

Table 4. *Oncorhynchus tshawytscha.* Results of histological analyses of kidneys from fish sampled at various times after injection or immersion challenge with *Renibacterium salmoninarum* strain MT 239 or strain 33209

Challenge type and treatment group[a]	Sampling date (wk post-challenge)	No. positive by IFAT/total tested	No. of fish with granulomas (no. of fish with F, M or D)[b]
Injection			
Control	1	0/3	0
	6	0/3	0
	10	0/3	0
	22	0/4	0
High-dose MT 239	1	0/3	0
	6	0/3	0
	10	0/3	0
	22	0/4	0
High-dose 33209	1	1/3	0
	6	0/3	0
	10	3/3	3 (1 F-M, 1 M, 1 D)
	22	4/4	4 (1 F-M, 2 M-D, 1 D)
Immersion			
Control	8	0/3	0
	22	0/4	0
High-dose MT 239	8	0/3	0
	22	0/4	0
High-dose 33209	8	0/3	0
	22	2/4	1 (F)

[a]Refer to Table 1 for bacterial concentrations of the treatment doses

[b]F = focal granulomas, M = multifocal granulomas, D = diffuse granulomas, F-M = focal to multifocal granulomas, M-D = multifocal to diffuse granulomas

Testing by the IFAT of histological sections from fish sampled at 1, 6, 10, and 22 wk post-challenge from the control and high-dose MT 239 groups detected no samples positive for *Renibacterium salmoninarum* (Table 4). At 10 and 22 wk, the fish sampled from the high-dose 33209 group all tested positive for *R. salmoninarum* with high numbers of bacteria (too numerous to count). The DFAT testing of the kidney-spleen homogenates from the fish sampled at 22 wk showed the same results as observed with the histological samples from these same fish (data not shown). Only the 4 fish from the high-dose 33209 group were positive for *R. salmoninarum*, and all had high numbers of *R. salmoninarum* that were too numerous to count (data not shown). At 22 wk post-challenge, *R. salmoninarum* was recovered in bacterial culture only from the fish sampled from the high-dose 33209 group (data not shown).

Immersion challenge

Fish examined from the control and high-dose MT 239 groups at 8 and 22 wk post-challenge did not exhibit BKD-related histological changes in the kidney, and all sections tested negative for *Renibacterium salmoninarum* by IFAT (Table 4). A focal granuloma was observed histologically in a fish sampled at 22 wk from the high-dose 33209 group, and 2 fish were positive by the IFAT (Table 4). Analysis of kidney-spleen homogenates by the DFAT from the same fish tested by histological examination showed that all 4 were positive for *R. salmoninarum* at 22 wk, but all had low numbers of *R. salmoninarum* (fewer than 10 *R. salmoninarum* per 100 microscope fields; data not shown). No *R. salmoninarum* were detected by the DFAT in the kidney-spleen homogenates from the control and MT 239 high-dose groups at 22 wk. Moreover, *R. salmoninarum* was not re-isolated in culture from the any of the fish sampled from the immersion groups at 22 wk (data not shown).

DISCUSSION

The results of this study showed a difference in virulence between the *Renibacterium salmoninarum* strains ATCC 33209 and MT 239 in chinook salmon *Oncorhynchus tshawytscha*. Attenuated virulence of MT 239 was demonstrated by both injection and immersion challenges. This is the first report of attenuated virulence of MT 239 in a salmonid species other than rainbow trout *Oncorhynchus mykiss*. It is also the first study in which fish were challenged with a virulent and an attenuated strain of *R. salmoninarum* via 2 exposure routes and analyzed for the presence of gross and histopathological changes. The attenuated virulence observed with chinook salmon corresponds with the reduced virulence previously reported in rainbow trout (Bruno 1988, Senson & Stevenson 1999). Therefore, the attenuated virulence of MT 239 occurs in at least 2 salmonid species: *O. tshawytscha*, which is highly susceptible to BKD, and *O. mykiss*, which is more resistant (Sanders et al. 1978, Bell 1987).

The results of gross observation and histological analyses showed that only the high dose of the 33209 *Renibacterium salmoninarum* strain produced pathological changes in the kidney tissues. The BKD-associated changes observed in this study have been previously described (Wood & Yasutake 1956, Young & Chapman 1978, Bruno 1986, Sami et al. 1992, Flaño et al. 1996). Fish exposed to the high dose of 33209 also exhibited changes in their melanomacrophage aggregates with individual melanin granules dispersed throughout granulomatous areas in the kidney tissue. The aggregates in the tissues of fish exposed to the control or MT 239 preparations remained as large dense deposits. The function of these aggregates is currently unknown, but some authors have speculated that the melanin granules aid the immune defenses through bactericidal action and that they may also have the ability to help protect tissues from harmful chemicals released by activated phagocytes (Agius 1985, Roberts 1989 p 30–31, 82–83, 137–143, 313–314, Wolke 1992).

The results of bacterial culture and DFAT with kidney-spleen homogenates indicated that MT 239 persisted in a few fish after the challenge even though this strain did not seem to cause BKD. The MT 239 strain was identified by DFAT in a few fish that died up to 11 wk after the injection challenge and in a few fish that died up to 20 wk after the immersion challenge. Viable MT 239 was re-isolated in culture only from a few fish that died up to 13 wk following the immersion challenge. Bacterial persistence was also reported by Senson & Stevenson (1999), who found that MT 239 was detectable by IFAT in at least one-third of the fish challenged by immersion but in none of the fish challenged by injection at 14 wk post-challenge. The study by Senson & Stevenson (1999) did not report whether MT 239 was identified in fish that died during the experiment and if the bacteria that were detected at the conclusion of the experiment (14 wk) were viable.

Some fish exposed to high doses of 33209 in the injection and immersion challenges may have cleared *Renibacterium salmoninarum* infections during the experiment. This could explain the observation that only 60% of fish that were tested from the high-dose injection and 40% from the high-dose immersion 33209 challenges were positive for *R. salmoninarum* by DFAT at the end of the experiment (22 wk after

challenge). One study has shown that *R. salmoninarum* antigen levels measured in various groups of infected chinook salmon decreased in some groups over time (Pascho et al. 1991). Another study reported that some fish exposed to a high-dose injection of *R. salmoninarum* survived to the end of the challenge study and showed no signs of BKD, indicating that all fish exposed to high doses of *R. salmoninarum* may not develop BKD, or alternatively, they may survive infection and then later show no signs of the disease (Bruno 1988). Nevertheless, no mechanisms for clearance of *R. salmoninarum* have been identified. These may be the same mechanisms that are effective against MT 239.

In groups of fish challenged with *Renibacterium salmoninarum* by immersion, variability in mortality was observed between tanks; this seems to have resulted from co-infections of *R. salmoninarum* and a *Flavobacterium*-like organism that caused caudal fin erosion. The affected fish had clinical signs of both the *Flavobacterium*-like disease as well as BKD. These fish may have already been exposed to the suspected *Flavobacterium* prior to this experiment as other fish (not in this experiment) from this stock were also observed with the same clinical signs. This organism may have been vertically transmitted from infected broodstock; evidence indicates that *Flavobacterium psychrophilum* can be carried within salmonid eggs (Brown et al. 1997). Several fish that were highly infected with *R. salmoninarum* in the high-dose 33209 groups also had the *Flavobacterium*-like infection, suggesting that the synergistic effects of both infections may have resulted in mortality. Fewer fish in the control and MT 239 groups had the *Flavobacterium*-like infection and fewer in these groups died, suggesting that the *Flavobacterium*-like infection alone may not have caused high mortality.

Among the fish that died, the single fish testing positive for *Renibacterium salmoninarum* by DFAT in a control group and about half of the fish testing positive for *R. salmoninarum* in the MT 239 groups had low numbers of *R. salmoninarum*. Low numbers of *R. salmoninarum* (from 1 to 5 or 6 to 50) in kidney smears tested by FAT have previously been shown to be ELISA-negative in a study which compared the 2 detection methods (Meyers et al. 1993). Consequently, low numbers of *R. salmoninarum* detected by FAT may not represent high infection levels or clinically diseased fish. The ELISA and PCR detection methods were not used on these samples but may provide more sensitive methods of *R. salmoninarum* detection (Pascho et al. 1987, 1998). The ELISA used in this laboratory primarily detects p57 antigen and may need to be optimized for use with the MT 239 strain. No detection method has yet been developed to quantitatively distinguish levels of MT 239 and 33209 if both are present in the same experimental sample.

The results provided in this investigation and in the previous study by Senson & Stevenson (1999) are the only reports to date examining salmonids exposed to the MT 239 strain by an immersion challenge. Both studies report the persistence of MT 239 following the challenge, but the reason for this persistence is currently unknown. In contrast to the intraperitoneal injection challenge method, exposure by immersion allows MT 239 to encounter several different portals of entry (e.g. gills, gastrointestinal tract, or skin) that may contribute to the ability of the bacteria to enter and become established within the host. These portals reflect those that *Renibacterium salmoninarum* must face during natural infections. Virulent strains of *R. salmoninarum* subvert the salmonid immune system and become established within the host despite the host immune response. For example, phagocytic cells in the skin may engulf *R. salmoninarum* but not kill them as *R. salmoninarum* reportedly survives within macrophages (Gutenberger et al. 1997). Thus, the bacteria are protected from other immune responses (cell-mediated). One *in vitro* report indicated that the MT 239 strain survived for at least 3 to 4 d within macrophages isolated from rainbow trout, which may explain the persistence of this strain within fish (Bandín et al. 1993). Challenging fish with MT 239 by injection may expose the bacteria to different aspects of the immune response than those encountered during an immersion exposure. Further studies with the MT 239 strain could examine other organs and tissues to investigate the fate of this bacterium following challenge by the 2 different types of exposure.

There are several possibilities to account for the difference in virulence between MT 239 and 33209 including possible differences in growth rate. *Renibacterium salmoninarum* normally has a slow growth rate requiring 7 to 10 d for observable turbidity in broth culture, and 4 to 6 wk for formation of observable colonies on agar plates. Thus, slow growth is a known characteristic for this bacterium. Growth rates for the 2 strains do not differ significantly in bacterial culture (Senson & Stevenson 1999). Nevertheless, the MT 239 strain may have a slower growth rate *in vivo* and may be easily detectable only at high levels which may have occurred beyond the last sampling time point in the experiment. The 2 previously reported MT 239 challenges monitored fish for only 5 or 14 wk (Bruno 1988, Senson & Stevenson 1999); the present study, however, followed mortality through 22 wk but did not detect a proliferation of MT 239 in the kidneys of challenged fish. Unless MT 239 requires longer than 22 wk to proliferate to a detectable level in fish, the slower growth hypothesis is unlikely.

Another reason for the attenuated virulence of MT 239 may be the difference in levels of p57 associated with the bacterial cell as 33209 has much higher levels than MT 239, but comparable levels of p57 are found in the culture supernatants of both strains (O'Farrell & Strom 1999, Senson & Stevenson 1999). The reason for this difference in p57 is currently unknown but may result from genetic differences (as yet unidentified) between the 2 strains. The p57 protein is a putative virulence factor, and although its specific role has not been characterized, its presence on the bacterial cell surface may be required for virulence. For example, it may be important in macrophage interactions, host cell adherence, or invasion. Furthermore, MT 239 may elicit a more protective immune response, which may allow the host to be more successful in eliminating this bacterial strain. This idea is supported by the work of Wood & Kaattari (1996), which showed that fish exposed to *Renibacterium salmoninarum* with p57 removed had a 20-fold increase in detectable antibody titers when compared to fish exposed to *R. salmoninarum* that had p57 intact on the bacterial surface.

The difference in virulence may also be due to a difference in macrophage interactions as virulent strains are phagocytosed, but not all of the bacteria are killed (Bandín et al. 1993, Gutenberger et al. 1997). Differences may occur in macrophage activation, phagocytosis, escapement from the phagolysosome, or intracellular survival. However, an *in vitro* study by Bandín et al. (1993) reported similar short-term survival for both virulent and avirulent *Renibacterium salmoninarum* strains (including MT 239) within rainbow trout macrophages. Additional studies examining different aspects of macrophage interactions with MT 239 would be useful. Future studies of fish exposed to MT 239 followed by challenge with a virulent strain would also be useful in developing vaccines against *R. salmoninarum*. Results from these types of experiments could further explain the basis for the attenuated virulence of MT 239, and the continued investigation of the mechanisms of attenuation could lead to a greater understanding of *R. salmoninarum* pathogenesis.

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